

Time for determination and hope in Russia

A new mood of optimism can be found in many parts of Russia's scientific community that has been absent for much of the past five years. But it must not be allowed to turn into complacency; many hurdles remain.

No longer is the talk in Moscow laboratories solely of declining salaries and broken government promises — or even, on occasion, suicide. The prospect of real change is in the air as plans for the reform of Russian science become more realistic, and the need for radical change becomes more widely accepted.

Part of the new optimism is based on the government's declared determination to introduce much-needed changes in the way Russian science is run, and to try to ensure that its budget is protected. A decree issued at the beginning of last month, for example, set out a number of important reform measures, including the promise to establish procedures for assessing the scientific performance of government research institutions, and closing those that fail to come up to scratch. And the science budget has been 'ring-fenced' against cuts during the year as an essential component of public spending. On past experience, government promises involving money should be viewed sceptically. The good news lies in its intention to focus resources more productively.

In this, the role of the Russian Academy of Sciences will be crucial. There are signs that the academy is prepared to grasp the nettle of change, albeit somewhat gingerly. Last week, it accepted pressure from the government by electing a number of 'young' academicians — meaning under 55 for full members, and under 50 for corresponding members — in an attempt to help attract new blood into influential positions in the research system (see page 535). More enthusiastic has been the academy's response to the government's decision that its individual institutes be allowed to rent out land and redundant facilities, if the income is used to purchase scientific equipment and to meet other infrastructure needs.

Hidebound

But too much of Russian science remains hidebound by outdated bureaucratic rules, as well as tensions and distrust between the state and much of the research community which, ironically for a country which has in the past invested so heavily in science, remains one of the deepest and most damaging legacies of the Communist era.

At the same time, new tensions have been created by some of the early reforms of the Yeltsin era. Many of these, such as the Russian Foundation for Fundamental Research and the now-defunct International Science Foundation set up by the financier George Soros, have attempted quite properly to establish new traditions of strict peer review, but have found it easier to do this outside the traditional channels. This has inevitably created tension with those who claim that the need is to reform the current system, not undermine it.

The result has been something of a backlash, with a shift in power back to the academy, and the dangers that this contains. Academy officials are correct to emphasize that the development of science in Russia, as indeed in any modern state, cannot be left to the private sector and market forces alone. What is needed now is a sustained and strategically targeted investment, coordinated but not controlled by the government, to build and maintain the country's scientific infrastruc-

ture. As the pressure on salaries subsides, for example, the main difficulty facing many laboratories, keen to meet international levels of scientific competence, is the absence of adequate research equipment.

As the government has recognized in last month's decree, achieving such targeted investment will mean some difficult decisions. The danger is that the academy will become excessively concerned with its own survival and the maintenance of its anachronistic system of privileges. As a result, it could well fail to find the courage to take what can often be painful courses of action, including the closure of unproductive institutes and the elimination of research groups whose output does not meet international standards.

Yet it is no longer sufficient for the academy to boast of its intellectual prowess on the basis primarily of its past achievements and quantitative measurements of published research papers. Money for investment in science is scarce in the modern world, perhaps in Russia more than anywhere else. It can afford to support only that which meets international standards of quality.

Continued support

To achieve this, Russian science needs continued support from the West. Here the efforts of the European organization INTAS, and the Civilian Research and Development Fund, set up under the broad umbrella of US/Russian cooperation agreements, continue to have an invaluable role to play.

But such actions are unlikely to achieve long-lasting change unless they are combined with political support for those reformers in government who are trying to introduce more flexibility, accountability and openness into the whole of the research system. Much was achieved in this direction by the former science minister, Boris Saltykov, who, before being replaced after last year's elections, sketched out a vision of how Russian science could rise again if — and only if — it was prepared to adopt practices that have proved successful in other countries.

The spirit of Saltykov's reforms lives on in the reforms introduced last month. One of the most important clauses is one that urges both the ministry of economics and the ministry of science, currently combined with telecommunications, to consult international financial organizations about the possibilities of investment in high technology and innovation. There is an important role for bodies such as the World Bank to help Russia to find ways in which it can tap into the bedrock of scientific talent which the country undoubtedly still possesses.

But the academy, too, must face up to the need for radical change. The academy's role as an arbiter of scientific excellence remains as important as ever. Less convincing is its continued conviction that it remains best placed to decide on the strategic distribution of scientific resources to meet the country's needs. Continued pressure from the government on the academy to adopt international standards and make tough resource decisions is needed. And continued international investment and collaborative scientific support from the West are required to help Russia tap the scientific talent that is now straining to express itself. □



New French government aims to boost research job prospects

[PARIS] Research and technology are likely to move up the political agenda in France following the victory of the Socialist party and its allies in last weekend's general election. But the government's room for manoeuvre will in practice be sharply curtailed by the overriding need to keep public spending in check in order to meet the Maastricht criteria for entry into European Monetary Union.

The Socialist party has not yet drafted a fully fledged manifesto for science, mainly because its plans to do so were pre-empted by the decision of Jacques Chirac, the French president, to call a snap election. But one likely outcome, according to Claudine Laurent, a member of the Socialist party's committee on science, is the immediate launch of initiatives to boost job opportunities for young researchers.

The government aims to ease the stagnation in recruitment that has occurred in the public research organizations over the past five years, in particular to offset the expected massive wave of retirement over the next decade; more than half the current research workforce is expected to retire by 2005.

But constrained by the need to limit public spending on the one hand, and keen to increase research in the private sector, the government also intends to encourage the recruitment of scientists by companies, especially the small and medium-sized. One idea is to link recruitment directly to the tax incentives given to companies that carry out research, worth about FF4 billion (US\$696,000,000) a year.

It is still far from clear how research will be organized at government level. Some French newspapers have predicted the creation of a superministry of industry, higher education and research, headed by Claude Allègre, a researcher at the Institut de Physique du Globe de Paris. But Allègre is being widely tipped as science minister primarily because he is a close friend and principal adviser of Lionel Jospin, the first secretary of the Socialist party and new prime minister (see *Nature* 374, 666; 1995).

Sources close to the party say Jospin would be unlikely to refuse Allègre a ministerial portfolio should he ask for it. But they point out that while Allègre is widely respected as an intelligent and staunch defender of research and education, his bruising, singled-minded style runs contrary to the consensual government promised by Jospin. Nevertheless, there is general agreement in Paris that Allègre is guaranteed a key post, perhaps as adviser to Jospin or as head of one

of France's major research agencies.

Nor is the creation of a superministry a foregone conclusion. One option raised frequently at a series of *ad hoc* meetings held over the past few weeks by the Socialist party and leading scientists and research administrators is the need to separate education from research. They were brought together in one ministry in 1993 by the then prime minister Edouard Balladur. But there is general agreement that the heavy day-to-day demands of the school and higher-education portfolios has led to a neglect of research.

A major challenge facing the new government is widely seen to be the development of a coherent national strategy for research in consultation with the various actors involved, and many scientists remain sceptical about its ability to do this. Another is tackling the long-standing problem of the

large proportion, often more than three-quarters, of the research agencies' budgets taken up by salaries. This leaves little money for the strategic programmes that many feel are needed to reinvigorate French research.

The coming months are also likely to bring changes in the individuals responsible for running the research agencies. Guy Aubert, director-general of the national research agency CNRS, finishes his current mandate in July, and Claude Griscelli, the director-general of the national biomedical research agency INSERM, is partway through his mandate. Although new governments traditionally allow existing mandates to expire, many observers predict they may make an exception for Griscelli, who is an associate mayor for health in Paris in a town hall racked by financial scandals.

Declan Butler

See also: synchrotron siting row, page 539

Russian academy elects younger blood



[MOSCOW] The Russian Academy of Sciences is, like its headquarters building in Moscow (above) being given a facelift. In addition to filling more than 100 'natural' vacancies resulting from the deaths of former members over the past three years in elections last week, the government had provided money for 50 additional full members younger than 55, and 100 new corresponding members younger than 50.

But, partly as a result of the controversy created by this move — some members had argued that scientific achievement should be the sole factor — not all the vacancies were filled. Only 61 new full members (including 16 'young') and 189 corresponding members (83 'young') were elected.

Further controversy was created by some political figures seeking election. Those successful included Mikhail Kirpichnikov, who heads the cabinet's department of science and education and who, it is said,

was responsible for the government's decision to help 'young' scientists (like himself) to be elected to the academy.

Others appointed to full membership included Viktor Mokhailov, the minister for atomic energy, and Viktor Sadovnichy, the rector of Moscow University. But Andrey Kokoshin, first deputy minister of defence, failed to win sufficient support, even though he was the only candidate for the vacancy.

Other political figures who failed to be elected included Alexander Shokhin, deputy speaker of the State Duma (Russian parliament), Alexey Egorov, deputy minister of science and technology, and three active politicians, Sergey Glaziev, Alexander Arbatov and Viktor Shevelukha.

Among those elected was Alexander Solzhenitsin, the writer and former dissident, who was recognized for his contributions to the enrichment of the Russian language.

Carl Levitin

Germany rejects genome data 'isolation'

[MUNICH] The German research ministry is expected to reverse its controversial decision to grant companies three months' privileged access to human genome sequence data generated with public funds, because of fears that German scientists could be isolated by the international scientific community (see *Nature* 387, 111; 1997).

The agreement on privileged access was made last year between the ministry and a new German industrial funding association, the Verein zur Förderung der Humangenomforschung. It angered scientists in other countries, particularly the United States, because it contravenes an informal agreement to make sequence data from publicly funded human genome-sequencing centres immediately available on the Internet.

At a meeting between the ministry, scientists and industry in Bonn last week, all sides agreed that the threat by geneticists in the United States and Britain to exclude German scientists from international collaborations in the Human Genome Project, and to block their access to vital biological material, outweighed the advantage to industry of the relatively short period of privileged access that had been agreed.

"We don't want to uncouple ourselves from the international scientific community," says Knut Bauer, head of the life sciences department in the research ministry. Rudolf Balling, head of the mammalian genetics department at the National Research Centre for Environment and Health in Munich, agrees. "Germany must be integrated with the international research community, as there is no such thing as a German Human Genome Project," he says.

But although the immediate conflict over the German rules seems to be over, many aspects of the complex issue of data release

remain unresolved. Bauer says that negotiations between industry and scientists will continue over the next month to find a compromise that will satisfy all sides. No one is willing to comment on what form such a compromise might take.

At the heart of the conflict lie differences between US and European patent laws, which industry, as well as the ministry and some patent experts, believes puts Europe at a competitive disadvantage.

Raw sequence data need extensive — and time-consuming — analysis to identify genes and their functions. European patent laws exclude patents on any gene whose sequence has already been published. In contrast, US patent laws offer scientists a grace period of one year after publication in which to seek a patent.

German industry wanted a period of privileged access to buy the necessary time for analysis, before German scientists lost their right to patent sequences by publishing them on the Internet.

Joseph Straus, a patent lawyer who chaired the Bonn meeting last week, says that while German industry may compete for patents in the United States, this would encourage companies to shift their research and development there, a trend the German government wants to reverse.

Feelings on the issue of the release of sequencing data have been running very high in Germany. André Rosenthal, a department head at the Institute for Molecular Biotechnology in Jena, who is coordinator of the German human genome-sequencing effort, had threatened to refuse his approved ministry grant of nearly DM20 million (US\$12 million) to set up his sequencing laboratory unless he was allowed to publish his data immediately on the Internet.

In particular, Rosenthal feels that German industry is paying too little for the privileged access it was demanding. "If industry wants to gain from sequence data", he says, "it should make the necessary heavy investment in-house."

The industrial association in Germany has been taken aback by the strength of feeling, but representatives say that it is eager to compromise with Rosenthal and others sharing his views. Thomas von Rüden, head of molecular biology at Boehringer Ingelheim's Vienna-based research and development centre, the Institute of Molecular Pathology, says the agreement with the ministry appears to be in line with the Bermuda agreement, which prescribes that publication should be "as fast as possible".

Having apparently won his case, Rosenthal is now campaigning on behalf of other ministry-funded scientists working in smaller laboratories who will still be obliged to offer to the industrial association sequence data from non-human species, and complementary DNA data. These are not covered in the Bermuda agreement, and he predicts that similar rows will break out in the future within these other related scientific communities.

But the Bermuda agreement covers only human genome sequence data generated at publicly funded, large-scale, high-throughput sequencing centres, such as that being built up by Rosenthal, which are intended as a service to a wide scientific community. Michael Morgan, a director of the Wellcome Trust, which funds the United Kingdom's large Sanger sequencing centre jointly with the Medical Research Council, believes that it is not necessarily appropriate for smaller laboratories to hook up to an Internet system of data publication.

However, he points out that a new trend for similar international agreements relating to pathogenic microorganisms of significant public health concern is gathering momentum.

Just as the row in Germany is about to be defused, a similar row in France may be imminent. The new, publicly funded Centre National de Séquençage in Paris, which will start operating in the summer, is at present debating its own rules about data release.

Its director, Jean Weissenbach, says a decision about whether to place all its data on the Internet or to grant French industry privileged access is due this month. The new German position may influence the decision in the direction of immediate publication, he says. But the outcome is not certain and, like Rosenthal, he fears the possibility of ostracism by the international community.

Alison Abbott

European ethics advisers back cloning ban

[PARIS] The cloning of humans for reproductive purposes is "ethically unacceptable", according to the nine-member group that advises the European Commission on the ethical implications of biotechnology. But, in an "opinion" issued last week, the group also stated that cloning animals is acceptable, provided it does not harm their welfare.

Widely viewed as a body set up by the commission to help adjudicate on disputes between the biotechnology industry and environmental

lobbies, the group has given few explanations of how it reached the conclusions of its seven-page opinion, which was agreed during a series of closed meetings (see *Nature* 387, 321; 1997).

The opinion recommends a ban on reproductive cloning of humans by nuclear transfer, arguing that "consideration of instrumentalization and eugenics" makes this unethical, and that such techniques would also be unsafe. It says that cloning by embryo splitting, "however

understandable" (see *Nature* 387, 324; 1997), should also be prohibited.

In contrast, the ethics advisory group says that research using cloning techniques should be permitted under strict licensing arrangements, provided that the research throws light on the cause of human disease or "alleviates suffering". But it adds that such research should not go as far as allowing the implementation of a manipulated embryo in the uterus.

Declan Butler

EU eliminates citation gap with America

[WASHINGTON] The United States remains the most scientifically productive region in the world — but only just. The member states of the European Union (EU) are about to take the number one spot, suggesting that Western Europe has regained its position as the world's leading producer of scientific knowledge.

At the same time, however, the quality of US science, at least as measured by the rate at which US papers are cited by other researchers, remains substantially higher than that of its main economic competitors, with the United Kingdom coming second, Germany third and France fourth.

These conclusions are based on analysis by the Institute for Scientific Information (ISI) in Philadelphia, and published in the May/June issue of ISI's *ScienceWatch*. They

Production of scientific papers, 1992–96

	Total no. of papers	Citations per paper
United States	1,239,188	5.03
United Kingdom	300,377	4.19
Japan	280,855	3.18
Germany	258,946	3.78
France	197,816	3.66
Canada	167,326	3.83
Italy	116,534	3.42
Australia	85,215	3.23
Netherlands	80,016	4.45
Spain	73,224	2.72
Sweden	61,072	4.38
Switzerland	55,213	5.66

Countries ranked by number of papers 1992–96
Source: Institute for Scientific Information

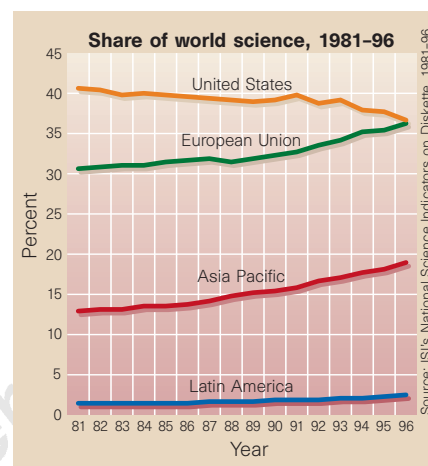
are based on citation statistics covering 102 subfields representing all areas of science, as well as the social sciences and humanities.

ISI's analysis reveals that between 1981 and 1996 the proportion of such papers with at least one author from the United States fell from 40.5 per cent to 36.5 per cent, with a particularly marked fall since 1991. In contrast, the proportion with at least one author coming from a member state of the EU increased from 30.5 to 36.2 per cent.

Europe's figures were, admittedly, bolstered by the fact that the number of countries belonging to the union rose from 10 to 15 during this period. The decline in the US lead also reflects the fact that the Asia-Pacific region increased its output significantly during this period — from 12.8 to 18.8 per cent of the total — as did Latin America.

But the new EU members are relatively small science-producers. The overall trend (see chart, right) indicates that, measured at least in terms of the quantity of scientific output, Europe has regained a role that it occupied from the scientific revolution of the seventeenth century up to the end of the Second World War.

In terms of scientific impact, measured by the number of citations to each of the papers published in the 102 specialist journals, Switzerland maintains the top ranking which it held in the previous comparative analysis, carried out by ISI in 1991. The Netherlands, Denmark and Sweden also remain in the top five — a reflection of the traditional high quality of the science in



these relatively small countries.

Among the larger countries, however, the United States remains ahead, recording an average 5.03 citations to each of its 1,239,188 published papers. "Although the United States has lost some of its world share of papers the overall strength of US science as measured by citation impact seems to be holding steady," comments ISI. The institute argues that the new figures appear to belie "grim predictions" about the state of US science at the time of the 1991 survey.

In terms of the relative strengths of different geographic regions in different scientific fields, the ISI analysis shows that the EU had a considerably higher-than-average citation rate in geological/petroleum/mining engineering, agriculture/agronomy, metallurgy and nuclear engineering. □

Europe seeks to head off oil-exporters' veto on climate treaty

[LONDON] European countries are making a bid to prevent members of the Organization of Petroleum Exporting Countries (OPEC) from blocking the signing of a new international treaty limiting the emissions of greenhouse gases that are believed to contribute to global warming.

The government of the Netherlands, acting on behalf of the 15 members of the European Union (EU), has tabled an amendment to the United Nations Framework Convention on Climate Change that would allow the treaty to be adopted by a three-quarters majority of countries if the parties fail to reach a consensus.

The requirement that such a treaty be backed by a consensus has put OPEC countries in a strong bargaining position (see *Nature* 378, 524; 1997). Their delegates are expected to oppose the proposed amendment strongly when it is discussed at the convention's annual conference in Kyoto, Japan, in December.

But EU governments are apparently

worried that, unless parties to the convention are able to vote on the treaty by majority, the oil-exporting countries and their allies could use the consensus requirement to block any proposals they disagree with.

Parties to the climate convention have already agreed to approve a treaty to reduce emissions of greenhouse gases such as carbon dioxide at the Kyoto meeting. Oil-exporting countries are demanding compensation for revenues they will lose if countries reduce their fossil fuel use to comply with the treaty. They are backed by the G77 group of developing countries, including India and China, and by at least one US fossil-fuel lobby group.

But observers such as Farhana Yamin, director of the Foundation for International Environmental Law and Development at the University of London, say that the treaty is unlikely to include such provisions.

Yamin says the EU proposal appears to have two goals. "It ensures that the adoption

of [the treaty] at Kyoto cannot be blocked by a small minority, and it weakens the bargaining position of such countries in the negotiations leading up to Kyoto."

Amendments to the climate convention normally need to be ratified by the parliaments of each signatory country before entering into force. This could potentially delay a vote on a greenhouse gas treaty for at least two or three years.

EU lawyers point out that the Vienna Convention on the Law of Treaties allows an amendment to come into force provisionally if it receives enough support, opening up the possibility of majority voting on a greenhouse gas treaty.

But one leading environment lawyer says that the proposed amendment could still be legally vulnerable, particularly if the treaty is approved by close to the required majority. "If one or two countries decided to change their minds afterwards, the entire treaty could potentially collapse," he warns.

Ehsan Masood

Microsoft 'in talks' to set up research base in Cambridge

[LONDON] Plans to set up a research centre at Cambridge in the United Kingdom to concentrate on long-term, strategic research leading to the next generation of computers are reported to be in the final stages of development by Bill Gates, founder and chairman of computer software giant Microsoft.

The centre would be owned and operated by Microsoft, but is expected to have close links to the University of Cambridge. Microsoft has had several meetings with university officials, including members of the university's council. According to some reports, one proposal is that academic staff teaching at the university should be allowed to carry out research at the Microsoft centre.

A spokesman for Microsoft says the company is "neither confirming or denying" the reports. But plans are believed to be well advanced. Alec Broers, vice-chancellor of the university, said that talks with Microsoft were "at an early stage". A statement released by the university on Monday (2 June) said that "we would be delighted if these talks result in some type of collaboration".

Two sites in the west of the town have been identified for the centre, and an official announcement is expected within a month. The plans follow a speech given by Gates earlier this year in which he revealed that Microsoft Research would be doubling in size within the next two years. Gates told the annual meeting of the American Association for the Advancement of Science in Seattle, Washington, that Microsoft's expansion plans were limited only by its ability to recruit talented people. The company spends \$2 billion a year on research and development.

The decision to establish a major research presence in Europe — in addition to the research facility in Seattle — appears to have been flagged during a speech Gates made to the World Economic Forum in Switzerland last year, in which he raised the issue of Europe's relatively low contribution to global software research. Various sites in Europe are believed to have been investigated for the company's planned move across the Atlantic before Gates settled on Cambridge.

Contacts between Microsoft and the University of Cambridge are believed to have been facilitated by Stephen Hawking, Lucasian Professor of Mathematics, who taught cosmology to Nathan Myhrvold, Microsoft's chief technology officer.

The university has recently launched plans to set up a physical sciences and technology research site, also in the west of Cambridge. Gates is also said to be planning to make a separate donation to the university to fund a new science block.

Ehsan Masood

Italian universities move to peer review for grants

[MUNICH] In a major shift in its university policy, Italy is to replace its much-criticized system under which national research grants are allocated by committees of 'experts' with a system based primarily on peer review and national strategic priorities.

In future, responsibility for national university grant allocation will lie in the hands of a small body of five academics, chosen by the research ministry. And, to encourage collaboration, government grants will be awarded only to joint projects between different research departments or universities.

These changes are part of a new law on universities that comes into effect next month. They are intended to bring procedures for supporting university research closer in line with international norms, and to increase competition between universities and between individual departments. But, while the reforms have been broadly welcomed, some researchers are concerned that the five-member panel will lack the breadth required to judge the whole spectrum of Italian science.

Italy's 60 universities receive relatively little support for research compared to universities in some other European countries. Under a law passed in the early 1990s, 60 per cent of the IL250 billion (US\$147 million) provided annually by the government for university research is distributed by the universities themselves, and the rest is distributed by Italy's national council for universities, the CUN (Consiglio nazionale universitario).

Under the present system, grant applications are judged by 14 committees — one for each discipline — each made up of about ten experts elected from the academic community. Although this system is democratic, there is a growing recognition that it fails to concentrate money on the most deserving projects.

In particular, each elected member is susceptible to lobbying. To minimize con-

flict, therefore, funds tend to be sprinkled evenly across the research community.

Under the new law, a single grant review committee will be established each year. It will have five members selected by Luigi Berlinguer, the research minister, from 15 names put forward by the Association of University Rectors, the government's scientific advisory group, the CNST (Consiglio nazionale scienza tecnologia) and CUN.

These five will select referees to judge each research application, and will be responsible for the allocation of grants based on a combination of referees' reports and national strategic priorities. At the same time, each of the 14 disciplines will be guaranteed 3 per cent of the total CUN research budget — a safety net to protect the overall health of Italian research.

The CUN grants will not fund entirely new projects, but will instead provide additional support for projects already receiving backing from local university research funds. Universities will thus have more control in pre-selecting projects.

Individuals will not be able to apply directly for CUN funds. Instead, a limited number of projects will be selected by local universities. Collaborations within a university will be entitled to a 40 per cent supplement to their local funds, while those between universities will be entitled to a 60 per cent supplement.

A spokesman for the research ministry says that referees will be chosen from a list of qualified individuals published on the Internet, and that the anonymity of the referees will eliminate the power of individual lobbies. The most important advantage of the new system, he says, is the way it encourages collaboration between research teams.

Some remain sceptical. Pierniggi Strata, a professor of neurophysiology at the University of Turin, says the new system ignores a key aspect of peer review. "The selection of referees should be done by experts in the field, not by drawing on a list of possible candidates."

Others are more optimistic. Alessandro Finazzi-Agro, a biochemist who is rector of the Tor Vergata University in Rome, shares Strata's concern, but feels that the new law will at least require universities to think more carefully about which applications they are prepared to support, and that the 'five wise men' of the new grants committee will be freer to make strategic selections.

According to Finazzi-Agro, the new system is an experiment, and "we'll have to wait and see how it works". In fact, the academic community has little time to debate its merits. The first call for grant applications goes out next week, with a deadline for submission next month.

Alison Abbott



Politics fuels French synchrotron row

[PARIS] The last days of France's outgoing conservative coalition government have been marked by a row over the siting of France's planned FFr1-billion (US\$174-million) 2.15-GeV synchrotron, with rumours that the government intended to forgo the formal selection procedure and site the facility in Bordeaux, whose mayor is the former prime minister Alain Juppé.

Following last weekend's election victory by the left-wing parties, Claudine Laurent, a member of the Socialist party's committee on science, says that the decision on the siting of the SOLEIL synchrotron will be reviewed from scratch.

Several cities are bidding to host SOLEIL. Among the favourites is Paris, whose region is already home to the LURE facilities (800 MeV and 1.85 GeV) — which SOLEIL is intended to replace — and which possesses the appropriate staff and technical experience, as well as a cluster of service companies.

At the same time, France is keen to decentralize its research (see *Nature* 356, 373; 1992), and strong contenders include Caen, Lille, Marseilles and Orléans — even though a new site outside Paris would greatly increase costs because of the need to hire new staff and build up local skills.

A late entry has been the city of Bordeaux. Both the research ministry and Matignon, the prime minister's residence, deny claims, first made in the newspaper *Libération*, that the government was considering choosing Bordeaux. But many feel there is strong circumstantial evidence to the contrary.

For example, although most candidates have spent months — if not years — preparing their applications, Bordeaux's was hurriedly put together within three weeks at the end of April.

Furthermore, siting bids for research facilities are normally submitted to the government by scientists and locally elected officials. But the Bordeaux bid was put in directly by the *préfet* of the region, who represents the government, and who called a meeting of local scientists in April in order to draw up the bid.

Last month's hurriedly called election has delayed an expected announcement by the government that it had approved the construction of SOLEIL. But the choice of a site is not expected until next year, as this requires a detailed assessment of the scientific and technical merits of tenders, as well as their feasibility and cost-effectiveness.

Although the final decision on big science facilities — which is formally the responsibility of the prime minister's office — tends inevitably to contain a substantial political component, scientists are keen that the assessment phase itself should not be rushed.

The National Union of Scientific Researchers last week condemned any premature decision to site the synchrotron in Bordeaux, claiming that this would be blatant political interference in the necessary consultation procedure. It urged the scientific community to "react immediately by letters, petitions and telegrams to the prime minister and president of the Republic" to



Where now? Former prime minister Alain Juppé is mayor of Bordeaux, which has bid for SOLEIL.

head off any such attempt.

"We wanted to put out the fire before it started, as it is always more difficult to stop afterwards," says François Wuilleumier, director of the Laboratory of Atomic and Ionic Spectroscopy at the Université Paris-Sud, one of the partners in the Ile-de-France region's bid for SOLEIL.

Indeed, Wuilleumier believes that the quick reaction of the scientific community made any premature choice of Bordeaux politically impossible. Concern has further subsided following the victory of the left in last weekend's general election, but some warn that vigilance is still required, as the presidential office could impose a decision.

One scientist who helped to draft the Bordeaux bid accepts that Juppé's presence has given Bordeaux a political edge on its rivals. But he argues that the project is scientifically sound, and points out that the Bordeaux dossier incorporates an earlier bid from nearby Toulouse, as part of a joint candidature for the Aquitaine region.

"An outsider knowing nothing of French politics would consider Bordeaux a strong candidate," he claims, arguing that the planned FFr6-billion 1.8-megajoule laser (see *Nature* 375, 6; 1995) near the city makes it the ideal choice as a 'pole' for high-power radiation research facilities.

Grenoble has already achieved this over the past twenty-five years, being home to the Institut Laue-Langevin neutron source and the European Synchrotron Radiation Facility, as well as a host of associated laboratories. One senior official at the Commissariat à l'Energie Atomique says, however, that it would be difficult to imagine siting an open civil facility such as SOLEIL next to the top-secret military megajoule installation near Bordeaux.

Declan Butler

France and Britain drop idea of joint machine

[PARIS] The prospect of France and the United Kingdom abandoning their planned national second-generation synchrotrons — SOLEIL and DIAMOND respectively — in favour of jointly constructing a single machine (see *Nature* 381, 100; 1996) has been definitively dropped.

David Norman, who took over this week as head of synchrotron radiation at the Daresbury laboratories in Cheshire, says that it has been agreed that the size of the user communities in each country justifies each building its own machine.

"Synchrotron radiation is now just part of the everyday tool kit of many scientists, and is no longer just the frontier research tool for the

few," says Norman, who took over from Ian Munroe, who has retired.

Instead, Britain, France and Switzerland — which plans to build the 2.1-GeV Swiss Light Source — will coordinate the construction of their respective synchrotrons to reduce costs and overlap. A tripartite agreement setting out the terms of such collaboration is about to be signed.

This will identify areas for cooperation, such as the joint design of components, the standardization of materials and procedures, and bulk purchasing. The agreement also addresses concern that the simultaneous construction of three large synchrotrons

could outstrip the capacity of European industry.

The deal will grant researchers reciprocal access to one another's beamlines. The 3-GeV DIAMOND machine produces harder X-rays than the Swiss and French machines, and is more suited to studying biological structures. Softer X-rays are better for determining electronic structure and spectroscopy.

The French government is expected to approve SOLEIL soon (see above), and the Swiss project is in the final stages of approval. John Cadogan, director-general of the UK research councils, this week submitted the full case for DIAMOND to the councils for comment. D.B.

Geologist loses 'creationism' challenge

[SYDNEY] Australia's 'creationism' trial ended dramatically last Monday (2 June) when the judge dismissed the main plank of the case that had been made by Ian Plimer, a geologist at the University of Melbourne, against Allen Roberts, a fundamentalist church elder who claimed to have found scientific evidence for the remains of Noah's Ark.

The verdict in the case, which had been closely watched in Australia and abroad, is likely to widen the gulf between scientists and Christian fundamentalists over biological evolution versus the literal truth of the biblical story of creation (see *Nature* 386, 529, 638 & 748; 1997).

Plimer and his US co-applicant, David Fasold, lost the case primarily because the judge rejected their argument that Roberts had acted in trade or commerce and had therefore illegally misled those who had provided him with financial backing.

But Judge Ron Sackville expanded this into a wider principle. "Courts should not attempt to provide a remedy for every false or misleading statement made in the course of public debate on matters of general interest," he concluded. "Some issues — no matter how great the passions they arouse — are more appropriately dealt with outside the courtroom."

Sackville accepted that Roberts had

infringed the copyright in a drawing of the supposed Noah's Ark by Fasold, a former believer in the existence of the Ark. But Fasold described the award of A\$2,500 (US\$1968) in damages as "a slap in the face", pointing out that he had won US\$40,000 and US\$42,500 in two US cases for similar breach of copyright.

After the verdict, Roberts said that he and his supporters had been "completely vindicated when it comes to this nonsense that's been set up under the Fair Trading Act". He claimed the verdict "preserved the free speech of anyone who has something important to say publicly", and hoped that the judgement would deter people from being "harassed and pursued through the courts" by someone "who disagrees with them ideologically".

But the judge had described as "false" Roberts's statements in lectures that he had carried out scientific tests on objects retrieved from the 'Ark' site in eastern Turkey. "Had the Fair Trading Acts applied, [these remarks] would have constituted misleading or deceptive conduct on Roberts's part," said Sackville.

In the five years before the court hearing, Plimer had devoted much time to presenting a public assessment of Roberts's ideas. Although Plimer has lost the latest round, he has already promised to fight on at a press conference held in more supportive territory — the fossil gallery of the Australian Museum in Sydney.

Vowing a renewed fight in public against "snake oil salesmen" and fundamentalist literalism which he sees as "a dark force in today's world", Plimer argued that free speech had been inhibited by the judgement and by the refusal of Roberts and other creationists to debate openly with scientists.

At a separate press conference, Roberts said: "I thank God that my colleagues and I are at last free." Casting doubt on Plimer's investigation of the supposed Ark site in 1994, he remains undeterred by scientific evidence. "The only way to settle the question is to get on the site with the Turkish scientists and archaeologists and others from all over and dig the thing responsibly and well," he said.

In his verdict, Sackville dismissed Plimer's allegations that Roberts's references to his doctorate in Christian education from Freedom University, Florida, as evidence of his academic qualifications in presenting archaeological results and conclusions constituted misleading or deceptive conduct.

Roberts's lawyers are preparing a submission for legal costs, likely to run to many hundreds of thousands of dollars. Plimer has stated that such costs would make him bankrupt. His solicitor says there may be grounds for appeal.

Peter Pockley



Plimer: faces high bill for legal costs.



Roberts: could face further actions.

HUNT-FAIRFAX PHOTO LIBRARY

US threat to end science agreement with India over patent law

[NEW DELHI] The United States is threatening not to renew its science and technology agreement with India if the Indian government fails to amend its patent laws to provide additional protection for intellectual property rights. About 130 projects could face the axe as a result.

India's current patent legislation, passed in 1970, does not allow patents in the food, chemicals and pharmaceuticals sectors.

After India joined the World Trade Organization in 1995, the government drafted legislation conforming to the organization's guidelines on intellectual property. But the proposed legislation has been blocked by the parliament's upper house, most of whose members come from opposition parties.

They argue that patents would make essential drugs too expensive for the poor, and allow seed companies to make agriculture too costly for marginal farmers. They also oppose patenting of any life forms.

Washington's warning of the potential consequences for collaboration in science and technology was conveyed to India by Frank

Wisner, the US ambassador in New Delhi. He said last week that both his government and US industry were "deeply concerned about the lack of adequate intellectual property rights protection provided by current Indian law, regulation and practice".

Wisner said that, until the situation is changed, the United States would be unable to negotiate a new science and technology agreement, and that "certain areas of research and training will be closed to cooperation". Wisner was speaking to senior Indian and US scientists at a meeting intended to draw up a strategy for future scientific collaboration.

The US decision has already generated strong reaction. India's Council of Scientific and Industrial Research, for example, which is responsible for about 40 laboratories and has agreements with 35 countries, said that it objected to "any conditions being placed for scientific and technological cooperation".

M. G. K. Menon, a physicist and former Indian science minister, says that any changes in Indian patent law should be made in a way that suits "our best national interests", and should not be dictated by the United States.

"We can do without US help," says Sandip Basu, director of the National Institute of Immunology in New Delhi.

Scientific cooperation between India and the United States has been operating in low gear since 1987, when differences about intellectual property rights first surfaced.

Roughly 130 projects, in different stages of completion, would have to terminate at the end of this year, when the US-India Fund, through which joint projects have been funded since 1987, will dry up. The US embassy in New Delhi says that the fund will not be replenished. The only project that would continue would be a vaccine research programme for which there is an assurance of continued funding.

Officials in the Indian ministry of science and technology are uncertain about the future shape of scientific and technological collaboration between the two countries. But they remain optimistic that collaboration will continue with individual US scientists and their funding agencies, where the work is carried out without the direct involvement of the US State Department.

K. S. Jayaraman

Weapons labs turn to computer simulation

Colin Macilwain

US nuclear weapons laboratories are moving to a regime based on linking experiment and computer simulation. But can they win over their critics?

The Science-based Stockpile Stewardship (SBSS) programme was conceived three years ago as a political compromise to keep the laboratories functioning and consolidate political support for the Comprehensive Test Ban Treaty within the United States.

The programme is now finally taking shape inside the laboratories, which have been deprived by the treaty of their historic function of developing new nuclear weapons, and were until recently uncertain of their future.

Although a programme to install the largest supercomputers in the world has only just started, scientists at the laboratories have already developed the first versions of entirely new weapons codes which will simulate existing nuclear weapons on the parallel processing computers. The codes replace far less powerful computer programs used to design weapons on older, vector-based machines.

New experimental facilities at the laboratories have not even been built yet. Federico Peña, the energy secretary, broke ground at a ceremony to mark the start of construction of the National Ignition Facility (NIF), the largest and most significant of these, at the Lawrence Livermore National Laboratory last week. But experiments at existing facilities are being used to feed new information into these codes.

Critical codes for simulation

Fuller understanding of the behaviour of the high-explosive, plutonium fission 'primary' and hydrogen fusion 'secondary' stages of nuclear weapons, and of their other components, will enable scientists at the laboratories progressively to refine these codes.

This prospect excites scientists at the weapons laboratories. But it alarms critics in the United States and abroad, who fear that the codes will be used to develop new generations of nuclear weapons, with or without nuclear testing (see *Nature* 387, 439; 1997).

Randy Christensen, a senior computer scientist at Livermore, says the new computer codes "are reaching the point where they begin to deliver results". He predicts that the laboratory, having lost its old rhythm in which computer-assisted design work led up to a climactic device test at the Nevada Test Site, yielding results to feed back into the design, will now establish a new rhythm.

Indeed, a major justification of NIF and the rest of the stewardship programme is the need to attract talented scientists to the weapons laboratories. Morale plunged among weapons scientists at Livermore and at Los Alamos and Sandia, the other weapons laboratories in New Mexico, after testing stopped in 1992. But several scientists at Livermore agreed with Christensen that it is already reviving as SBSS takes shape.

Last week's ground-breaking at NIF, a \$1.2-billion laser facility that will help to underpin Livermore well into the next century, should have capped the revival. But court action by an alliance of environmental groups opposed to SBSS means that actual construction work — originally due to begin in April — has now been postponed until 27 June at the earliest.

The issue is politically contentious. Despite protests at the ground-breaking, Ellen Tauscher (Democrat, California), the newly elected congresswoman for the district containing Livermore, said that "people here are overwhelmingly supportive" of NIF construction. But other Californian politicians kept away from the ground-breaking, as did the US vice-president Al Gore.

In summarizing the case for NIF, Peña cited the support of the veteran physicists Hans Bethe and Herb York. "I am absolutely convinced that NIF is required to allow us to enter into the Comprehensive Test Ban Treaty without losing our nuclear deterrent," he said, adding that his judgement was largely based on the advice of scientists who support non-proliferation.

Prominent in that group is Sidney Drell, deputy director of the Stanford Linear Accelerator Center (SLAC), whom the previous energy secretary, Hazel O'Leary, says convinced her of the case for SBSS. But two other prominent former weapons scientists and non-proliferation advocates — Ray Kidder, a Livermore Laboratory Associate (see *Nature*, 386, 645–647; 1997), and Richard Garwin, IBM fellow emeritus — have recently criticized aspects of SBSS.

Garwin and Kidder both support NIF as a science experiment. But they say that its ability to simulate conditions in the 'secondary' of a nuclear weapon has been oversold. At the same time, both hotly dispute the claim by Ted Taylor, a former weapons designer at Los Alamos and long-time anti-nuclear cam-



Breaking new ground: Energy secretary Federico Peña (centre) digs in at the ignition facility site.

paigner, that NIF is a proliferation risk that could be used to develop new types of nuclear weapon.

NIF will use 192 giant laser beams to fire a brief energy pulse of 500 terawatts of peak power at a tiny fusion target, called a hohlraum, possibly igniting a small, controlled, but self-sustaining fusion reaction.

This ignition has been the holy grail of fusion researchers for fifty years. Partial-power experiments are due to start late in 2001. Livermore scientists hold different views on how soon after that they should attempt to reach ignition.

Wider than weapons research?

Like the rest of SBSS, NIF is being paid for by the Congress as a weapons programme. But at the ground-breaking, much of the focus was on the possibilities it will offer to non-weapons scientists, including those from outside the Department of Energy laboratory system.

But NIF's main role is to serve as an anchor for the Lawrence Livermore National Laboratory — just as SBSS as a whole was conceived to support the three laboratories and their Nevada test site.

For proponents, this approach meets a critical national-security objective, attracting exceptional new talent to the weapons programme and retaining it for unknown future needs. For opponents, it is pork-barrel politics of the worst kind: the price to be paid, as Frank von Hippel, a former official in the Clinton administration, has put it, to win support of the weapons laboratories for an end to weapons testing.

Some time in the next eighteen months, the Clinton administration will need 67 votes in the Republican-controlled Senate to ratify the Comprehensive Test Ban Treaty. The administration hopes that the successful initiation of SBSS, and the flow of \$4 billion annually into the US nuclear weapons programme, will help to achieve it. □

Patent-searching used to assess novelty of research proposals

[PARIS] The European Commission in Brussels is testing the customized use of patent-searching techniques as a way of assessing the novelty of research proposals within its technology transfer programme.

A preliminary study of 100 proposals in the programme, known simply as 'Innovation', has classed 30 as innovative, but questioned the originality of 57 others. The test of the technique, known as Quick Scan, will now be extended to 1,000 proposals.

The research proposals were subjected to the standard novelty search as carried out by European Patent Office examiners, which involves a computer search of 30 million documents. Although a research proposal differs from a patent application, the commission claims that the use of customized procedures allowed the examiners to obtain "conclusive" results for 87 of the proposals.

Two projects budgeted at ECU500,000 (US\$575,000) have already been rejected as a result of the study, the resulting saving representing ten times the cost of the search.

UCSF decides on second site in San Francisco

[SAN FRANCISCO] After years searching for an appropriate site for expansion, the University of California at San Francisco has decided to build its \$800-million second campus on 43 acres of waterfront land in a southern industrial district of San Francisco.

The city of San Francisco, keen to keep the university's jobs and prestige close by, gave 30 acres as an incentive, while the developer Catellus Development Corporation contributed 13 acres and concessions on streets, water lines and sewers.

The university had endured years of legal wrangling and battles with neighbourhood groups as plans for one site after another fell through. Residents living near prospective locations complained about parking problems, toxic emissions, animal testing, and unchecked growth, among other things.

UK arrangements under scrutiny

[LONDON] Britain's new Prime Minister, Tony Blair, has promised to study whether the post of chief scientific adviser, a position occupied by Sir Robert May, should remain within the Department of Trade and Industry (DTI), and also whether there is a case for uniting political responsibility for research councils with that for university funding.

In reply to a parliamentary question last week from Robert Jackson (Conservative,

Wantage), a former minister responsible for science, Blair said that both issues were being examined as part of a broad review of government arrangements for the organization of science being carried out by John Battle, the minister of state responsible for science in the DTI.

The government has already confirmed that, at least in the short term, both May and Sir John Cadogan, the director-general of the research councils, are to remain in their present positions, and that the budget for the Office of Science and Technology, including the research councils, will remain 'ring-fenced' within the DTI. It is also said to have decided to maintain the previous government's decision to freeze science spending over the next two years.

Astrophysicists win 1997 Crafoord prize

[LONDON] The 1997 Crafoord prize has been awarded to the astrophysicists Sir Fred Hoyle and Edwin Salpeter for their "pioneering contributions" involving the study of nuclear reactions in the development of stars. The prize, estimated to be worth US\$500,000, is awarded annually by the Royal Swedish Academy of Sciences in recognition of outstanding basic research in fields not covered by the Nobel prize.

Hoyle, former director of the Institute of Theoretical Astronomy at the University of Cambridge, coined the term 'big bang' to describe the now widely-accepted theory explaining the origins of the Universe. Salpeter, professor of astronomy and physics at Cornell University, is known for the 'Salpeter process', which describes how older stars have energy that allows them to continue to shine.

Return to academia for EPA research head

[WASHINGTON] Robert Huggett, the marine scientist who has headed the US Environmental Protection Agency's research office since 1994, left this week to return to the College of William and Mary in Williamsburg, Virginia. Huggett says he has served his "allotted time" in the federal government and wants to return to research. "If I want to get back into academia, I can't stay away any longer," he says.

Brookhaven staff told they will lose tenure

[WASHINGTON] Six hundred scientific staff at Brookhaven National Laboratory on Long Island, New York, will lose the tenure arrangements that guarantee their posts when the laboratory gets a new contractor on 1 November. Staff were told of the change by Lyle Stewards, acting director, at a stormy,

three-hour meeting last week.

Staff members also received the required six-months notice of termination of their employment with Associated Universities Incorporated, which was dismissed last month as the Brookhaven contractor (see *Nature* 387, 114; 1997). But the US Department of Energy says that the new contractor "must agree to offer employment to virtually all Brookhaven employees at comparable pay and benefits, except for key management personnel" and will be a not-for-profit organization.

Austria seeks European view of proposals

[MUNICH] The European Science Foundation (ESF) is to assess the scientific and technical cases for two competing proposals for a new medium-size national research facility in Austria. One of the proposed facilities, Austron, would provide a pulsed neutron source for basic and applied researchers. The other, Eurocryst, would provide a laboratory for crystal synthesis and research.

The Austrian government and the Austrian Academy of Science have asked the foundation to provide an independent assessment of the proposals because of the ESF's ability to bring a European perspective to issues facing a small country. The report will be completed before the end of the year.

France may create genotyping centre

[PARIS] The French government has launched a feasibility study for a proposed national genotyping facility for multigenic diseases, to be built at the site of the Généthon laboratory at Evry, south of Paris. Dedicated to gene mapping and the localization of genes, the centre would extend Généthon's early landmark work in gene mapping and complement the planned FF1-billion (US\$172-million) national gene sequencing centre nearby (see *Nature* 383, 466; 1996).

Garden of Eden project gains lottery funding

[LONDON] The 'Garden of Eden' project, an ambitious scheme to create a series of domed greenhouses at a disused china clay pit in Cornwall, has been awarded £37 million (US\$61 million) from the proceeds of Britain's National Lottery. The domes, which will cover an area of eight hectares, will allow researchers to simulate a sub-Saharan desert, the vegetation of temperate and Mediterranean regions, and a rainforest (see *Nature* 379, 289; 1996). The scheme will cost £106 million, and is expected to attract 750,000 visitors each year. It is the brainchild of Tim Smit, a former record producer who restored a nearby Victorian garden.

Science as a cultural construct

Sir — In the days of sound-bite epistemology, I should not be surprised that Gottfried and Wilson¹ characterize my book, *Constructing Quarks*, by two short and decontextualized quotations from the last two paragraphs of a text which is 415 pages long (not counting a 43-page bibliography, most of it citations to the high-energy physics (HEP) literature)². One unfortunate consequence is that readers might imagine that there are no argumentative connections linking my “preposterous conclusions” to the historical substance of my book — which Gottfried and Wilson concede “displays a solid command of the developments that led to the Standard Model” and covers the “transition from the ‘old’ to the ‘new’ physics . . . very well indeed”, and which Gottfried has elsewhere generously described as “a superb account”³. I do have some arguments, however.

Gottfried and Wilson assert as self-evident that (a) the old and new physics were of “objectively unequal merit”, implying that (b) this was the reason that the latter eclipsed the former. In regard to (a), I show in *Constructing Quarks* that this was a matter for legitimate dispute. One can argue that the standard model fits some philosophical criteria for theory-choice better than the theories and models of the old physics, but conversely one can argue that the old physics had the merit of explaining almost all of the phenomena that appear in the HEP laboratory. New-physics data are terribly rare, and become visible only when an overwhelming ‘background’ of old-physics events is excluded from the analysis. Point (b) I believe to be historically untrue. In my historical research, I interviewed more than a hundred leading high-energy physicists and studied the published and unpublished literature extensively, and, in all honesty, that evidence did not persuade me that “objective merit” (as defined by Gottfried and Wilson) was what induced most physicists to move from one domain of knowledge and practice to the other. More positively, the same evidence did persuade me that such shifts could be easily understood in terms of what I called the dynamics of practice, relating research trajectories to the prior expertise of the physicists involved, symbiotic circuits of experimental and theoretical practice and so on. Much of *Constructing Quarks* is devoted to exemplifying that point; it is one of the links from the history to my “preposterous” conclusions; and neither Gottfried and Wilson, nor any other

particle physicist, has ever disputed the adequacy of that aspect of my book.

The other critical thread that runs through Gottfried and Wilson’s essay concerns the issue of prediction in science. They accuse me of assuming “the power to stop the clock at an arbitrary point, thereby ignoring subsequent evidence as to whether some bandwagon fell off the cliff or stayed on track”. The term bandwagon does not figure in my interpretive lexicon, but there is a grain of irrelevant truth to that quotation. I handed over the final manuscript of *Constructing Quarks* in mid-July 1983 (immediately after my daughter Lucy was born), so I could not, for example, follow the story of the discovery of the W and Z particles very far (though I did my best: see the long footnote 44, 379–380, dated July 1983). But I did follow similarly important stories that had more-or-less run their course before paternity took over — scaling, the weak neutral current, charmed particles (see also my other publications expanding on these topics: copies available on request). And for those discoveries I argued in detail, again, that my model of the dynamics of practice fitted the historical evidence better than simple assertions that ‘we got it right’. If Gottfried and Wilson want to challenge my specific accounts and interpretations of those events, I would be happy to argue with them. Failing that, it seems reasonable to regard their invocation of the Ws and Zs as a red herring.

One last remark. Gottfried and Wilson read into *Constructing Quarks* the sceptical message that “science is only a communal belief system with a dubious grip on reality”. But the message was not sceptical: I defy anybody to find textual warrant for either the “only” or the “dubious” in that quotation. Like many scientists (and philosophers), Gottfried and Wilson assume that *Constructing Quarks* asserts the opposite of their stock image of science, but this is just a mistake. Over the past fourteen years I have explored more deeply the engagement of science with its object, the material world; for that, see my latest book, *The Mangle of Practice*⁴. But nothing there contradicts what I wrote in *Constructing Quarks*.

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Sir — During its first quarter-century, discussion of the sociology and history of scientific knowledge (SSK) was largely restricted to social scientists. We welcome the growth of interest from natural scientists, especially as the most recent contributions represent a move towards serious debate.

We think we are still divided by rivers of misunderstanding, but at least they are no longer oceans. We can now see the possibility of building bridges. On our side we are beginning to see that our way of putting things, conditioned by 25 years of debate within the social sciences, might give natural scientists cause for concern. At the same time, we seem to be making progress in showing that most of this concern is misplaced. We will content ourselves in this letter by trying to clarify a few points.

Those who describe themselves as sociologists of scientific knowledge agree about most things. There are minor ‘philosophical’ disagreements between those who were first trained at Edinburgh and others, such as us, but the case-studies that emerge under the SSK label are virtually indistinguishable. ‘SSK’ is the most useful description for the group who consider that the core of the subject is careful, sociologically informed case-studies of experimentation and theory building.

SSK does not try to compete with natural science in the establishment or evaluation of scientific findings. We are social scientists and we do not think that evaluation of scientific findings is our business except in special circumstances; such circumstances have not arisen in this debate.

Because we are not in the business of evaluating scientific findings, we think it would be wrong of us to offer a running assessment of the pieces of scientific knowledge we discuss. Gottfried and Wilson¹ suggest that when we discuss some scientific finding we should keep the reader up to date with the latest developments in the field.

It is as though they would argue that a historical study of what made people give up phlogiston and take up the idea of oxygen would be flawed unless it referred to the way oxygen is presented in the periodic table, used by divers and welders, and saves lives in hospitals. We are concerned with what caused changes in view before things were thoroughly established because we want to use this as a model of what happens in current controversies.

We believe that the most constructive discussions will turn on the question of contemporaneous versus retrospective history of science. The purpose served by

each approach needs to be drawn out. We look forward to developing the analysis alongside our new colleagues.

H. M. Collins

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Trevor Pinch

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Sir — We welcome the tone of Gottfried and Wilson's assessment of what they (not we) call 'Edinburgh school' sociology of scientific knowledge¹. Their eschewal of the *ad hominem* attacks that have characterized too much of the 'Science Wars' debate is refreshing.

Three points of clarification are, however, in order. First, the goal of the sociology of knowledge, in our view, is the explanation of belief, not its evaluation. 'Symmetrical' analysis of the emergence, development and acceptance or rejection of bodies of knowledge does not involve the (absurd) claim that all beliefs are of equal merit. Rather, 'symmetry' implies that current evaluation of the truth or falsity of beliefs should not bias the empirical study of the processes through which knowledge

develops. For the historian or sociologist studying nineteenth-century evolutionism, for example, both Darwinism and anti-Darwinism stand equally in need of explanation.

Our second point is a simple corollary of the first. Predictive success is, of course, a vital measure of the merit of bodies of knowledge, and an important cause of their acceptance or rejection. But later predictive success cannot be appealed to as a cause of earlier acceptance.

Third, we have always held that satisfactory analysis of the development of knowledge must be multicausal. Causal input from the real world and psychological and social processes are all involved. Scientific knowledge is not a collective fantasy devoid of relation to the real world, but neither is it a simple mirror of reality. The sociology of scientific knowledge stands or falls by the value of the best, empirical, case-studies of these complex questions. We are glad that these case-studies are attracting the attention, however sceptical, of distinguished natural scientists. We benefit from their scrutiny; perhaps they may learn something in return.

David Bloor

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Sir — Gottfried and Wilson¹ criticize the Edinburgh school of sociology's contention that scientific knowledge is a communal belief system with a dubious grip on reality. The assessment is based on two points: (a) the neglect of a large body of experimental evidence that constrains scientific knowledge and guides theory and (b) the predictive power of science.

There is another powerful element that exposes the faulty logic of the 'Edinburgh' school: technology. Science constrains possible technology. When the latter (a new device, instrument or system) becomes reality, it validates the specific theories on which it is founded. If scientific knowledge was not an objective description of reality, but merely a social construct, we would not have technological realities such as transistors, microprocessors, personal computers and the Internet, or semiconductor lasers, fibre optics and compact disc players in addition to radios, cars and jets.

It is instructive to examine a few of the thousands of 'food chains' connecting scientific knowledge (the product of hypotheses and experiments converging into a theory) to technology.

Consider lasers and in particular semiconductor lasers, an essential element of long-distance fibre-optic communications. Without the concepts of

confine itself to the creation of practice and knowledge. It also makes sweeping, and at times flamboyant, Last Judgements about scientific practice and knowledge as a whole, as in the concluding chapters of *The Golem*⁵ and *Constructing Quarks*². It is this aspect of the SSK literature that is our first concern, for these judgements can be 'understood' by laymen and schoolchildren, whereas the technical parts of the case studies cannot.

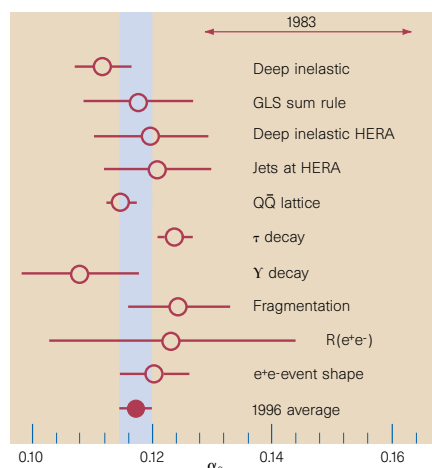
As to prediction, of course "later predictive success cannot be appealed to as a cause of earlier acceptance". We made no such appeal in bringing up chaotic motion in the Solar System and the Planck spectrum of the cosmic background radiation, which were discovered long after classical mechanics and the quantum theory had become firmly accepted. They are among many examples that illustrate the astonishing (and mysterious) ability of science to account for phenomena of which no inkling was available when the relevant theories were first accepted. The SSK literature is biased because it does not include case studies of such episodes, nor of experiments that test established theory to high accuracy.

Rather, it devotes great attention to two other topics: first, to the undeniable fact that scientific practice and knowledge rarely conform to any known set of philosophical ground rules; second, to features of scientific knowledge that are common to knowledge as a whole. Such commonalities abound; scientific knowledge is generated in ways that have typically human characteristics. But the end product is very different from those of virtually all other human activities.

SSK argues that scientists make choices contingent on culture. There is truth to this. But if that were all — if there were as much freedom to choose as SSK claims — a good portion of the major theories whose acceptance had been successfully 'negotiated' should fail disastrously in regimes where they were supposed to work, as popular policies often do in a democracy. Why scientific knowledge is, in contrast, so robust is not explained by the canon of SSK.

Collins and Pinch say that the "evaluation of scientific findings is not our business" (a position they share with Bloor and MacKenzie). Pickering, in contrast, evaluates the findings of particle physics in part as a former professional particle physicist with his own unusual scientific views (see pages 250–252 of ref. 4).

Constructing Quarks is a "best, empirical, case-study", to use Bloor and MacKenzie's term. Nevertheless, having been active participants in the move to the new physics, we disagree with Pickering's conclusion that "objective merit" was not



There were two errors in the figures in Gottfried and Wilson's article¹. The corrected Fig. 1 (above) illustrates the coupling constant (α_s) of QCD as determined today from various experiments and calculations, compared with the range of values determined in 1983. The term "Fragmentation" was incorrectly labelled "1983". In Fig. 2, the green line referred to in the caption was incorrectly printed blue.

"what induced most physicists to move". Indeed, consider the following statement in Pickering's account (see page 411 of ref. 2): "...the world of the old physics was conceptually and socially fragmented. Traditions organized around different phenomena generated little support for one another.... With the advent of the new physics, the conceptual unification of forces was accompanied by a social unification of practice. The quark-gauge theory world view was at the heart of a community-wide symbiosis of experiment and theory."

As our essay explained, we agree with an edited version of this statement; the old physics was fragmented because there were few theoretical connections between its various models and recipes, whereas the new was a powerful theory that provided a unified account and unambiguous predictions, some of which were quickly confirmed. Pickering means his statement to be read as saying that unification was primarily a social phenomenon, "a communal search for a congenial world: a world in which practice could be socially organized", and it is this claim that underlies his final verdict against the objectivity of modern science.

Finally, to Capasso's excellent letter. We set technology aside because we could not do justice to it in the space allotted. Technology plays an essential role as both input and output in the processes of continuous improvement of scientific practice and knowledge after a theory is born, a topic to which SSK gives little notice. This is part of a larger problem we often encounter in the SSK literature: an

inadequate description of the culture of physics — of the conceptual, historical and technological context in which research in modern physics is actually done.

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Patents and royalties

Sir — Philippe Ducor points out in his Commentary (*Nature* **387**, 13–14; 1997) that due to "the wise licensing policy of non-exclusivity and modest pricing adopted by the owner (Stanford University), [recombinant DNA] technology was widely distributed and contributed enormously to the subsequent development of commercial biotechnology. However, the outcome would have been radically different if Stanford had chosen instead a restrictive licensing strategy... or granted no licences."

This is true. But this policy was set carefully so as to generate the largest possible return without causing the patent to be contested. Companies wishing to use the technology weighed the cost of royalties against the cost of a suit and eventually all decided it was cheaper to pay royalties. If one of the other options suggested had been taken, it is likely that the patent would have been contested and there is a significant probability that the patent would have been ruled invalid.

Stanford and the University of California have already gathered hundreds of millions of dollars in royalties on this basic patent, despite the "modest pricing". The work that gave rise to this technology and its key patents was paid for by ordinary people through their tax dollars used for basic research. Ordinary people have subsequently had to pay many times over the original investment through patent royalties. Should people be penalized for having spent tax money wisely in the first place?

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Words and rules in the human brain

Steven Pinker

A new study of neurological patients who can analyse regular past-tense forms of verbs but not irregular ones, or vice versa, suggests that the mental dictionary and the mental grammar may be kept in different parts of the brain.

Regular and irregular verbs, the bane of every language student, would seem to be an unlikely route to understanding the brain. But they have spawned an intense debate in psychology, linguistics and artificial intelligence for over a decade. Researchers are now trying to resolve the debate using data from cognitive neuroscience, and on page 592 of this issue¹, William Marslen-Wilson and Lorraine Tyler report that some neurological patients can process one type of verb, but not the other.

Most verbs in English form the past tense by suffixing *-ed*: for example, *walk* becomes *walked*. When people encounter a new verb such as *to out-Yeltsin*, or a nonsense verb such as *wug*, they readily supply a regular past-tense form: *out-Yeltsined*, or *wugged*. These new forms could not have been memorized, so they must have been generated by a mental operation along the lines of 'add *-ed* to the verb'. Even four-year-old children do it, and the past tense has long served as the textbook demonstration that symbol-processing rules, such as those underlying grammar, are implemented in the human brain.

Regular verbs have a natural control group — the irregular verbs, such as *ring-rang*. There are only about 180 irregulars, but they are among the most common verbs in English, and they are unpredictable in form: for example, compare *ring-rang* with *bring-brought*. People seldom generalize irregular patterns to a new verb, so they would say that a car got *dinged*, rather than *dang* or *dung*. Apparently, irregular verbs are stored in and retrieved from memory, just like other words, rather than being generated by a rule^{2,3}.

This neat division between generative rules and memorized exceptions (the word/rule theory) has been contested by the school of cognitive science known as connectionism. Connectionists point to a kind of artificial neural network, called a pattern-associator memory. In their classic computer model⁴, David Rumelhart and James McClelland used a bank of input units for sounds in the verb stem, a bank of output units for sounds in the past-tense form, tunable connections between every input and output, and a learning mechanism that recorded correlations among stem sounds and past-tense sounds (for example, *-ing* with

-ang, and *-alk* with *-alked*). The model learned the past-tense forms of several hundred verbs, and generalized with fair accuracy to new verbs. Its errors resembled those made by children (such as *come-comed*), but it didn't use anything that looked like a rule, nor did it have distinct components for regular and irregular forms. So connectionists claim that symbolic rules, and any qualitative distinction between

regular and irregular verbs, are fictions⁴.

But evidence from several recent studies fits a key prediction of the word/rule theory — that different parts of the brain are involved to different extents when people process regular and irregular inflections. Michael Ullman and his collaborators found that patients with agrammatic aphasia (a difficulty in composing sentences that is usually caused by damage to the left anterior cortex of the brain) have more trouble inflecting regular and novel verbs than irregulars⁵. By contrast, patients with anomia aphasia (a difficulty in retrieving words, usually caused by damage to left temporal and posterior cortices) have more trouble with irregular verbs.

The techniques of functional magnetic resonance imaging and positron emission tomography have been used to record metabolic activity in the brains of subjects forming past tenses. These studies have found that different regions of the brain are active for regular and irregular verbs⁶⁻⁸. Another experiment

Remembrance of things past: three theories

The debate on regular and irregular verbs centres on the distinction between symbolic rules for grammar and an associative memory for words. What are the major theories?

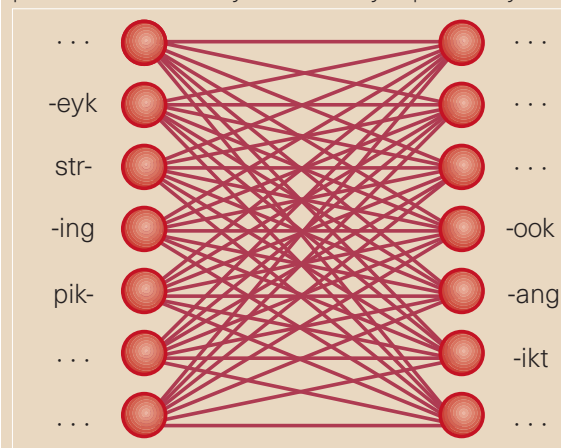
Rules all the way down. The linguists Noam Chomsky and Morris Halle¹² proposed that both regular and irregular forms are generated by rules. For example, *i*→*a* for *sing*, *ring*, *spring*; *ey*→*oo* for *take*, *shake*, *forsake*; verb→verb + *-ed*. The theory explains how people (including children) can produce a past-tense form for any

verb, even if it is new or unlike those already learned. It also explains why irregular past forms mostly resemble their stems (*take-took*, *sing-sang*), and why a given vowel-change pattern tends to be found in many verbs (*take-took*, *shake-shook*, *forsake-forsook*).

Associative memory all the way up. In the Rumelhart-McClelland⁴ model (and 15 subsequent variants), correlations between the sounds of stems and past-tense forms are superimposed in a large set of connection weights (see figure). The theory explains why

families of irregular verbs do not fall into well-defined classes, but are only statistically similar, and why people generalize irregular patterns to new verbs only if they are very similar to old ones; for example, *bring-brang*.

A rule for the regulars, memorized words for the irregulars. The Chomsky-Halle and Rumelhart-McClelland theories were combined by myself and others^{2,3,13}, rooting regular verbs in the combinatorial grammar that is needed for sentences, and rooting irregular verbs in the memorized lexicon that is needed for words. The theory combines the advantages of the rule-only and memory-only theories, as well as explaining how verbs with the same sound can have different past-tense forms (for example, *ring/rang* the bell versus *ring/ringed* the city). Marslen-Wilson and Tyler interpret their neuropsychological data as support for this theory. S.P.



recorded electroencephalographic activity (event-related potentials) in response to words in German-speaking subjects. Irregular nouns bearing a regular suffix elicited signals that were typical of the brain's reaction to syntactic violations, whereas regular nouns bearing an irregular suffix elicited signals that were typical of the reaction to anomalous words^{9,10}.

Marslen-Wilson and Tyler¹ now document the dissociation between the response to regular and irregular verbs in a new way. When people hear a word, they are 'primed' to recognize related words. Just after hearing *swan*, for example, people recognize *goose* more quickly, because the entries in their 'mental dictionary' overlap, or are linked. According to both the pattern-associator and word/rule theories, the irregular *find* should be associated with its past-tense form, *found*, in the same way — and, indeed, *found* primes *find* just as *swan* primes *goose*.

The regular *walked* primes its stem, too. But, according to the word/rule theory, it is for a different reason: the brain unconsciously analyses *walked* into *walk* and *-ed*, and the stem — *walk* — primes itself. (The priming is known to be caused by grammatical relatedness, not mere overlap in sound, because overlapping but unrelated words, such as *gravy* and *grave*, do not prime each other.) Marslen-Wilson and Tyler predict that if regular and irregular priming are based on different mechanisms, then neurological patients with different defects might have one or the other missing. They present a spoken priming word followed by a spoken target word, and the patients are asked to decide whether the target is a real word or a nonsense word. The patients do not have to speak aloud, eliminating the worry that irregular forms are more easily pronounced than regular ones. And the technique taps a rapid, unconscious process, so any difference in performance is unlikely to arise from special strategies that subjects concoct to carry out the task.

The new results confirm that the priming processes for regular and irregular verbs are neurologically distinct. In two patients with agrammatic aphasia, *found* primed *find* and *swan* primed *goose*, but *walked* did not prime *walk*. Presumably their circuitry for grammatical analysis is impaired, yet the associations in their mental dictionaries — such as *swan* to *goose* — are not as impaired, and these associations also embraced the links between irregular forms and their stems. In a third patient, the dissociation went the other way: *walked* primed *walk*, but *swan* failed to prime *goose* and, as expected, *found* failed to prime *find*. The authors interpret the evidence as support for the word/rule theory, and a challenge for connectionism: in a pattern-associator memory, the representations of regular and irregular inflections are superimposed in a single matrix of connection weights¹¹.

A simple explanation for the findings in terms of the patients' patterns of damage

might run like this. The first two patients (who lost priming of regular verbs) have massive damage to the left hemisphere — including the areas for grammatical processing — but no damage to the right hemisphere, which contains partial knowledge of words and their meanings. The third patient (who lost priming of irregular and semantically related words) has large damage to the right hemisphere and patchy damage to the left. So perhaps both copies of his mental dictionary are damaged, but just enough grammatical machinery has survived to analyse the regular forms. Marslen-Wilson and Tyler have not tried to advance such an explanation, possibly because the damaged region in each patient is diffuse and known only roughly; the role of the right hemisphere in associating words is controversial; and the third patient's ability to analyse regular verbs at the same time as having difficulty with other grammatical forms is difficult to explain convincingly.

Unfortunately, it is premature to look at the neuroimaging experiments to pinpoint the functions. They differ in methodology and in their exact findings. Furthermore, some of the components of the past-tense-generation task — remembering the verb and the tense, applying the suffix or retrieving irregulars, and suppressing the inappropriate form — may be neighbours in the brain, and hence difficult to distinguish.

More generally, we are largely ignorant of how language is computed in the brain, even with the recent stunning advances in neuroimaging. Cognitive neuroscientists must get the whole person to behave, and any bit of language behaviour must recruit many

abilities at once: words, grammar, meaning and knowledge, intentions to speak or believe what is spoken, and short-term memory for earlier parts of a sentence. It is difficult to devise a task that varies the operation of one component while holding the others constant. The recent flurry of studies on the neurology of the past tense — aside from its contribution to the connectionism debate — may offer hope for a better understanding of language and the brain in general. Irregular and regular verbs are nicely matched in complexity and meaning; and regular inflection, which people compute so freely when faced with new verbs, is perhaps the simplest example of the great human capacity for generating an unlimited number of new linguistic forms. Perhaps regular verbs can become the fruitflies of the neuroscience of language — their recombining units are easy to extract and visualize, and they are well studied, small, and easy to breed. □

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Quantum mechanics

Quantum procrastination

P. T. Greenland

Much of the practical business of quantum mechanics is in calculating exponential decay rates of unstable systems. Exponential decay is a familiar property of any system whose decay rate depends on its remaining, undecayed quantity (as in the decay of a large number of radioactive nuclei). But on page 575 of this issue¹, Wilkinson *et al.* present evidence for non-exponential decay — a clear prediction of quantum mechanics that has not been observed² until now.

This apparent paradox is reasonably well understood³. Quantum mechanics allows us to calculate the 'survival probability': the probability that a nearly bound state prepared at time $t = 0$ will still be found at some later time t . This probability falls as a function of time; the state decays. It is possible to show from the equations of quantum mechanics that for both 'short' and 'long' times this fall is slower than an exponential

function of time. For 'intermediate' times, the survival probability is exponential to a high degree of approximation, although it is still possible to show that (perhaps unobservably small) deviations from an exponential law must occur.

These results are independent of detailed features of the interactions that cause the decay of the unstable system, and require for their proof only two very general properties: there must be a lower bound to the energy that the decay products can have, and the decaying state must have finite energy. The detailed interactions then determine what is meant by 'short' and 'long'.

Three timescales are important⁴ (we shall not be concerned with the fourth timescale, at which long-time deviations from exponential decay occur). First is $\tau_w = \hbar/\Delta E$, where ΔE is the bandwidth of the continuum, the range of energies over which coupling is large between the unstable, nearly

bound state and the continuum of decay products. This is the memory time of the continuum. The phase information required for the reconstruction of the initial state is preserved on this timescale, and the survival probability starts to fall more slowly than exponentially (it falls quadratically). A second timescale is $\tau_E = \hbar/E_0$ where E_0 is an energy characteristic of motion in the initial nearly bound state; τ_E is the timescale on which the decay products separate. Often, τ_E and τ_W are comparable, but if $\tau_E > \tau_W$, the decay law in this region can be very complicated.

It is only for times larger than τ_W and τ_E that exponential decay starts. This decay proceeds on a characteristic timescale τ_D , the inverse of the usual exponential decay rate. It is set by the strength of the coupling between the nearly bound state and the continuum at the energy of the nearly bound state.

In almost all cases, the timescales τ_W and τ_E are so small ($\sim 10^{-17}$ s in atoms and 10^{-23} s for nuclei), that we have no chance of observing them. Wilkinson *et al.*¹ designed their experiment with this in mind. They first trap sodium atoms in a magneto-optical trap, and cool them using standard techniques, to leave $\sim 10^5$ cold atoms. These atoms are then caught in a standing light wave formed by two counter-propagating lasers. The laser fields provide a periodic potential in which the atoms move.

The quantum motion of the atoms in this potential is well understood; they fall into sets of stable energy bands. If the lasers have the same frequency, the standing wave is stationary, but if a frequency shift is introduced, the standing wave pattern accelerates. Atoms that are swept along with this acceleration are separated from those that cannot follow it. In the accelerated frame the standing wave pattern and the energy bands are tilted, so an atom can tunnel to a different position, and a different band, without changing energy.

In this experiment three accelerations are used. First a gentle one, which all the atoms in the lowest band can follow. This separates a group of atoms that are all in the lowest band. That is followed by a more violent acceleration which not all these atoms can follow, so some are left behind. Finally, those remaining in the lower band are separated from these stragglers by another gentle acceleration. The whole ensemble is then irradiated by the lasers used for the magneto-optical trap. That freezes the atomic motion and induces fluorescence, which allows the position of the atoms to be determined. Those that started in the lowest band and also followed the violent acceleration can be identified, from which the survival probability of the lowest band can be determined.

It is possible to analyse the dynamics in terms of a tunnelling process between the tilted bands. Atoms that fail to follow the

strong acceleration tunnel out of the lowest tilted band with a rate that can be calculated; we expect the survival probability to fall off exponentially with the duration of the strong acceleration, and indeed, that is what the same group had previously found⁵. They have now been able to measure the short-time behaviour of this decay, and it is not exponential.

Two features of the new experiment enabled it to measure pre-exponential decay. First, the experimental technique closely mirrors the theoretical idealization. In essence, a state is prepared, and coupled to a continuum by an interaction which lasts for a specific time. The survival probability over this time can then be determined. Second, although τ_E (the timescale for the internal motion of the atom in the optical lattice) is determined by the acceleration, the tunnelling rate (which determines the expo-

nential decay timescale τ_D) is set by other properties of the system as well. So it is possible to choose both the duration of the pre-exponential region — here about 5 μ s — and the decay rate in the exponential region, to show both features in the same experiment.

It will be interesting to see whether this set-up can be used to observe the quantum Zeno effect⁶ — the inhibition of decay by repeated observation in its very early stages, near τ_W . If so, we shall finally have confirmation that the adage ‘a watched pot never boils’ is indeed true, and a consequence of modern physics. □

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Molecular systematics

The platypus put in its place

David Penny and Masami Hasegawa

The duck-billed platypus, an egg-laying mammal, seems a contradiction in terms — not surprisingly, Richard Owen, a leading nineteenth-century anatomist, refused to believe the first reports from Australia that a platypus in captivity had laid eggs, because the animal is otherwise largely so mammal-like. In a formal sense, the conflicting features were resolved by placing monotremes (platypus and the related spiny anteaters, the echidnas) as the earliest mammalian lineage, pre-dating the later divergence of placental and marsupial (pouched) mammals.

This presumed relationship has now been thrown wide open with strong evidence from DNA sequences, published by Janke and colleagues^{1,2}, showing that monotremes are placed within these other mammals, specifically on the lineage leading to the marsupials. The new results are coming from a rapidly expanding number of complete mitochondrial genomes sequenced from a wide variety of vertebrates and invertebrates. The largest number are from mammals, and mostly come from the Arnason laboratory in Sweden^{2,3}. Instead of having DNA sequences only a few hundred to a thousand nucleotides long to analyse, the mitochondrial genomes have 16,500–17,000 nucleotides for each of more than 30 vertebrate species. Suddenly this standard, or long nuclear sequences⁴, has become the new minimum for studying vertebrate relationships.

A year ago there was a surprise when the platypus mitochondrial genome was reported by a German–Australian group¹, and it turned out that it joined firmly with the marsupial lineage, not earlier in the evolu-

tionary tree as almost universally expected. But there are always potential flaws in any analysis of this kind, and it was important to sequence the mitochondrial genome of a member of another taxon to break up the long branches to the platypus or to the first marsupial sequenced, the North American opossum *Didelphus*. The mitochondrial DNA of an Australian kangaroo species (wallaroo, *Macropus robustus*) has now been sequenced² — again, the study strongly supports the position of the platypus on the marsupial lineage (or the marsupials on the monotreme lineage) (see Fig. 1 overleaf).

To be a bit technical, the authors² used all standard methods to build evolutionary trees and test their result, including maximum likelihood on amino acids with a rate matrix derived from mitochondrial proteins⁵. All approaches give the same marsupial/monotreme relationship. The statistics seem to be sound with respect to monotremes, and the length of the marsupial/platypus branch is significant under maximum likelihood. The next best alternative places the platypus on the early placental-mammal lineage; least likely is the traditional view of the platypus occurring before the divergence between marsupial and placental mammals, and resampling methods reject the ‘monotremes first’ hypothesis 95 per cent of the time. The oldest divergences among placental mammals have percentage support values in the high 90s.

The new figure shown here is based on 19 complete mitochondrial genomes, using a log-determinant correction⁶ that allows unequal frequencies of amino acids to be taken into account. The amino-acid compo-

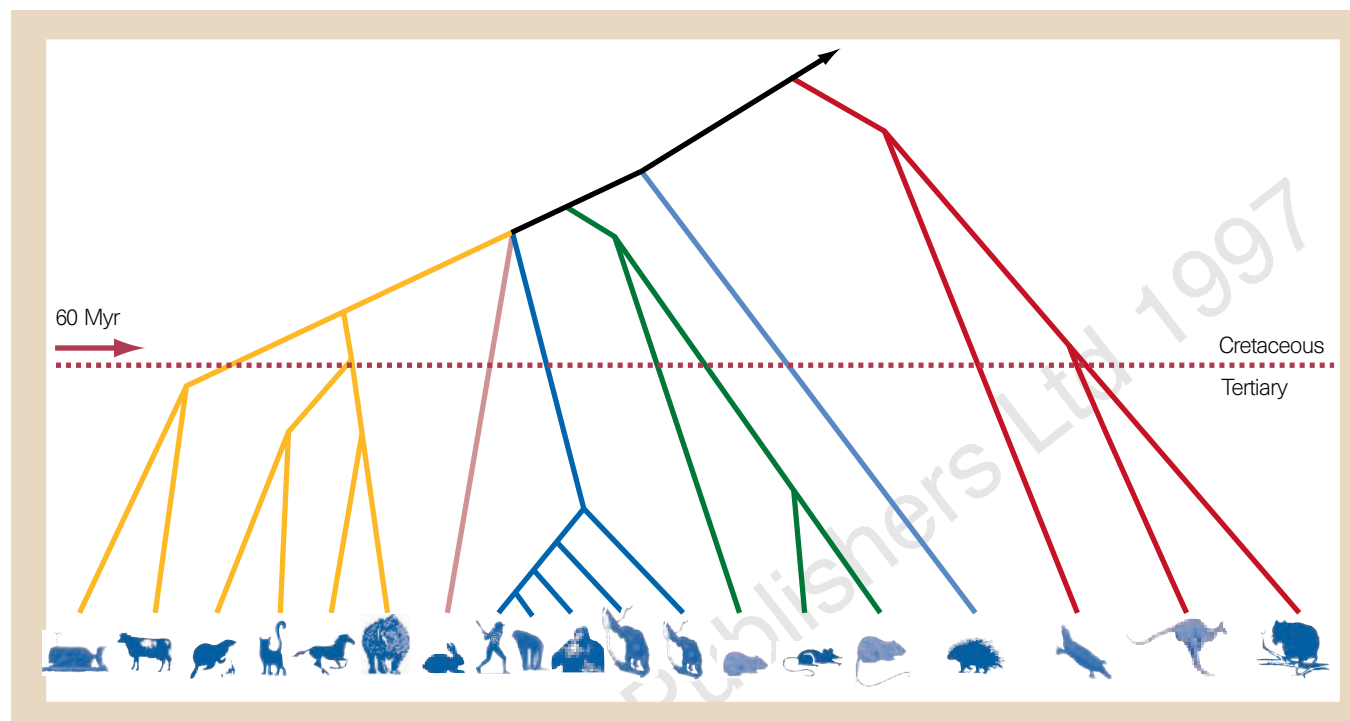


Figure 1 Combined evolutionary tree for 19 mammals, based on 12 proteins encoded by the vertebrate mitochondrial genome. In particular, it reflects the new results of sequencing^{1,2} that place the platypus on the lineage leading to marsupials, and not in a position pre-dating the divergence of marsupial and placental mammals. Because of the acceleration of evolutionary rate in mammals, the relative times of divergence can only be estimated for placental mammals. Approximate timings are shown based on a cow/whale divergence at 60 million years ago¹¹. Species included are, from left to right: whale, cow, seal, cat, horse, rhinoceros, rabbit, human, chimpanzee, gorilla, orangutan, gibbon, guinea pig, mouse, rat and hedgehog (all placental mammals); platypus (a monotreme); wallaroo and opossum (marsupials). As regards other features, the position of rabbit is uncertain, but close to the divergence of ferungulates (yellow) and primates. The edentates (sloth, armadillo and anteaters) appear to join early on the ferungulate branch¹². An African group⁴ (including elephant, hyrax, sea cow, elephant shrew and golden mole) may also join close to the ferungulate/primate divergence, but its precise position is still unresolved.

sition of the 12 proteins coded for on the same DNA strand varies significantly. But the platypus DNA is fairly average in its amino-acid composition, and has no extreme values for any amino acid, so unequal amino-acid compositions cannot account for the result.

The platypus story is not simply a debate over molecules versus morphology for determining evolutionary relationships, because one of the leading mammalogists of this century, W. K. Gregory, strongly championed his palimpsest theory that the origins of the monotremes lay within the marsupials⁷ (his Marsupionta hypothesis). This was no casual suggestion, but a major publication summarizing a lifetime of study. Gregory considered that monotremes have "so many deep-seated mammalian characters of the brain, milk, glands, etc." that they could only be considered true mammals, not precursors of mammals.

Gregory pointed to morphological similarities to the marsupial koalas and wombats (phalangers), and explained the apparently unique features of the platypus as follows. The rhinarium (nose region) of phalangers was enormously enlarged and modified to become the platypus beak; the reproductive system he considered as an arrested (neotenuous) stage of development that was transient in some marsupials; and he suggested that many unusual or unique features (not

shared by echidnas) are specializations to an aquatic habitat.

Why the difference in interpretation of morphology? Perhaps traditional idealistic morphology has regarded Australian mammals as 'primitive' in not having developed the 'advanced' features of other mammals. The more recent approach⁸ (as well as that of Gregory) is to consider the main characteristics of Australian mammals as adaptations to an environment which, because of prolonged droughts, is a harsh one for warm-blooded animals requiring a regular food supply. Survival over droughts lasting several years is aided by slightly lower body temperatures (especially when not active) and reduced brain size. Carnivores would be at a particular disadvantage during long droughts, and until the human occupation of Australia many of the continent's carnivores were reptilian⁸.

Progress will now be rapid, with mitochondrial DNAs from other vertebrates becoming available and other laboratories becoming involved in sequencing. After 200 years of research, the main outline of the mammalian radiation should be resolved within the next five years (perhaps to within one step on the evolutionary tree⁹). Moreover, a range of other questions can be addressed. What are the times of divergence of the main avian and mammalian orders, and how do these compare with the timing

of the breakup of the supercontinent Gondwana^{4,10}? How many land vertebrates survived events at the Cretaceous-Tertiary boundary⁹? When did mammals 'reclaim the day' (as opposed to being mainly nocturnal, or arboreal)?

It is necessary to integrate molecules, morphology, geology and ecology into an understanding of vertebrate evolution. Solving the evolutionary tree will not be the end of the study; rather it will be the start of many new investigations that will define the principal factors that shaped vertebrate evolution. □

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Evolutionary biology

Is speciation no accident?

Roger K. Butlin and Tom Tregenza

New species arise when previously conspecific populations no longer interbreed successfully, even when they are living in the same place. This reproductive isolation can often be explained as an incidental by-product of other evolutionary changes¹. But whether natural selection ever acts directly to increase isolation has been a controversial question ever since Dobzhansky² championed the idea in 1937. The proposal is straightforward — when populations have diverged genetically to the extent that the offspring of within-population matings are more fit than hybrid offspring, selection favours an increase in assortative mating (non-random mating resulting from a preference for similar partners). Assortative mating increases reproductive isolation, reducing the exchange of genes between the populations.

Despite the undoubted appeal of taking some of the chance out of the origin of species, this mechanism — known as reinforcement — has been challenged theoretically³, and it has never been supported by fully convincing examples. However, the idea has recently been undergoing something of a renaissance, and it will be boosted by a new study from Sætre *et al.*⁴, published on page 589 of this issue, which makes a case for reinforcement in European flycatchers.

A standard test for reinforcement is to study mating signals or mate choice in an area where the ranges of two populations overlap (sympatry), and to compare these with the mating behaviour of each population where it occurs alone (allopatry). Reinforcement should result in greater

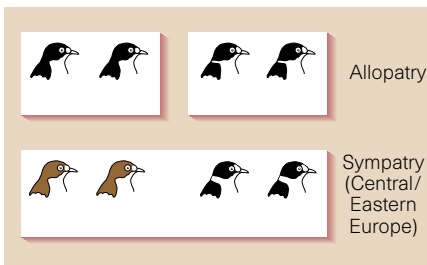


Figure 1 An increase in assortative mating can lead to the development of a new species, in a process known as reinforcement. Sætre *et al.*⁴ now provide a convincing example of this with their study of two species of European flycatcher. The ranges of *Ficedula hypoleuca* and *F. albicollis* are mainly separate (allopatry), and males have a similar black-and-white plumage. But in Central and Eastern Europe, where the ranges overlap (sympatry), the two species appear much more distinct.

divergence, or stronger assortative mating, in the area of overlap. Unfortunately, there are other possible explanations for this pattern³, so a convincing example must also show that the pattern evolved *in situ*, in the face of gene exchange, and that it reduces the frequency of unfit hybrids. Sætre and his co-workers have come closer to this than any previous study, with their work on the European *Ficedula* flycatchers.

The principal players are the pied flycatcher (*F. hypoleuca*) and the collared flycatcher (*F. albicollis*). The two species are allopatric over much of their distribution, and males have similar black-and-white plumage. However, where their ranges over-

lap in Central and Eastern Europe, the pied males are brown rather than black, and the collared males have extended areas of white, making the two forms much more distinct (Fig. 1).

Sætre *et al.* argue that this divergence is due to reinforcement, by demonstrating four points. First, between-species (heterospecific) matings are rarer than expected, and the hybrids generated do have reduced fitness (roughly 30% of parental). However, they produce enough offspring through matings with parental types to allow regular gene exchange. Second, a phylogeny that is based on mitochondrial DNA sequences indicates that the divergent plumage of the sympatric population is a separately derived character in each species, strengthening the inference that it evolved *in situ*. Third, females from sympatric populations choose to mate with males of their own species, and they do so much more reliably when the plumage colour of the males is of the more distinct, sympatric type. Finally, when given a choice between two males of their own species, females from sympatric populations prefer males that have the sympatric colouring over those with the allopatric colouring. This is especially remarkable for the pied females — their preference for dull, brown, sympatric males over the more striking black-and-white allopatric males (Fig. 2) is the opposite to the preference for conspicuous males shown in most populations, which is thought to be based on sexual selection for male quality⁵.

The last two points show that the combination of plumage traits and female preferences is enough to explain the reduced production of hybrids. At present, both the phylogeny and the data on mate choice are based on limited sampling. The case for reinforcement will be more secure when the generality of the preferences has been established, and when a more complete picture of the biogeographic history of the flycatchers is available.

The new study is more satisfying than another proposed example of reinforcement, in *Drosophila*⁶, because the signal traits involved have been identified. More fundamentally, the studies differ in the levels of gene exchange that are observed between the two species in each case. Although there is hybridization between the two species of *Drosophila* (*D. pseudoobscura* and *D. persimilis*), it is so rare — and hybrid fitness is so low — that the two genomes can remain distinct in sympatry. This weakens one of the main theoretical objections to reinforcement: namely, that recombination can break down associations between the genes that are involved in mate choice and those contributing to selection against hybrids, removing the advantage to assortative mating. If isolation is already strong, as in the *Drosophila* example, the reduction in recombination between



Figure 2 Birds of a feather. The plumage of the pied flycatcher (*Ficedula hypoleuca*) is black and white in areas where the birds are allopatric (for example, in Wales; left panel), but dull brown where the birds exist in sympatric populations with the collared flycatcher (*F. albicollis*) (for example, in Central Europe; right panel).

MIKE LANE

JUAN M. SIMON

species may make reinforcement much more likely⁷. The flycatcher case is different because 2.6% of matings produce hybrids, and their fitness (around 30%) allows plenty of opportunity for gene exchange. Of course, if reinforcement has occurred it must have initially operated against much higher levels of interbreeding, and it would be interesting to measure the extent of mixing of neutral alleles between the sympatric flycatcher populations.

Comparative studies that show much stronger behavioural isolation between pairs of sympatric *Drosophila* species than between allopatric pairs^{8,9} also support the reinforcement hypothesis. More recent models^{7,10} tend to show higher probabilities of reinforcement than do older models, mainly due to more realistic views of the

operation of mate choice⁷ and the genetics of hybrid dysfunction¹⁰. We may not be returning to Dobzhansky's whole-hearted enthusiasm for the idea, but it does seem to be much more plausible now than it was only a few years ago. □

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Archaeology

Husbandry at the oldest henge

Elizabeth Aveling

Henges are ancient circular monuments formed by an outer mound and an inner ditch, generally dating from the late Neolithic (3000–2000 BC). As possible sites of ritual activity, they replaced the causewayed camps of the early Neolithic (4000–3000 BC). Causewayed camps, which are usually associated with long barrows (earthen burial mounds), were also circular constructions, with concentric ditches segmented by several causeways, whereas henges are continuous earthworks except at their entrances. Henge monuments, with one or two exceptions, are specific to the British Isles, and there are several hundred of them. A henge in northern England has now been shown to pre-date Stonehenge by some 800 years¹. Its unusual shape could shed new light on the origin and significance of these enigmatic structures.

The purpose of henge monuments is unknown. Were they open-air temples for the worship of astronomical bodies? The site of markets for the exchange of goods and cattle? A ritual meeting place where people could come together to find marriage partners? Or a combination of these?

The Coupland enclosure in the Milfield Basin, Northumberland, has been known from aerial photographs for several decades (Fig. 1), but recent excavation of the site, by Clive Waddington and his team from Newcastle and Durham universities, has thrown up a few surprises. The site is described in a new booklet¹.

Henge monuments are not unusual in the archaeologically rich Milfield Basin², but the Coupland enclosure is unique in several ways. Its first unusual feature is its sheer size: with a diameter of approximately 110 metres, the area enclosed by the mound and ditch is more

than 19 times greater than other henge sites in the area, which are all about 25 m across. Two carbon-14 measurements of charcoal³ date the site to between 3800 and 4000 BC. This places the site in the early Neolithic, and makes it the earliest henge-like enclosure².

The most exciting discovery, however, is a double-ditched linear feature, bisecting the enclosure between its two opposed entrances, and continuing for 1.7 km to a ford on the River Till. This 'droveway', as it has been called, has no parallels at other henge sites. Pottery found along it shows that it is broadly

contemporary with the henge. Post holes to either side of the ditches at the entrance to the enclosure are all that remain of a gateway to the henge. The depth of these holes indicates that the gate was a chest-high affair, of similar dimensions to a modern farm gate. There is also evidence that the section of droveway closest to this entrance was fenced with wooden planks standing to a similar height⁴. On the basis of this evidence, Waddington has interpreted the Coupland enclosure as a cattle kraal for the over-wintering of livestock. We know that cattle grazed the uplands in summer but were driven into the valley for winter — what better place to drive them to than a ditched kraal in the middle of the settlement belt? Phosphate analysis of soil samples taken from the droveway, apparently unaffected by later activity, are currently being undertaken at Bradford University by my colleagues; high phosphate levels would imply an accumulation of organic material.

The cattle kraal theory is a nice, neat, sensible one. The work at the site has been thorough, involving intensive field walking, aerial photography, geophysical surveying, phosphate and pollen analyses, and so far the evidence seems to imply that the henge really was used for the management of stock. Cattle could have been brought down from the uplands that flank the basin to the west, south and northeast, and driven into either end of the enclosure. There is no recorded evidence for stock kraaling at other henge sites — but it has never been looked for before. And although causewayed camps contemporary with the Coupland henge have evidence for the killing and eating of cattle, they have never been considered as stock kraals either. ►



Figure 1 For farming or festivals? The Coupland enclosure in northern England is the oldest-known henge monument, nearly 6,000 years old, and a unique 'droveway' runs between its two entrances. It was probably used to keep cattle.

So, does this rather mundane explanation mean we have to discard our view of Stonehenge as an ancient temple in exchange for one of an elaborate livestock control system? Probably not. There may still have been elements of ritual behaviour at the Milfield site. The Neolithic inhabitants of the basin lived in small groups, probably with their own agricultural plots. Certainly, the presence of 'inscribed grazing areas'⁵ (defined areas of upland containing clear concentrations of cup and ring engravings) suggests that different communities may have claimed areas of pasture as their own. The Coupland enclosure in the valley, on the other hand, is likely to have been a neutral zone, and there is no reason why people should not have met here to exchange commodities as well as to over-winter their cattle. Perhaps the very coming together of different groups in one special area designated it as sacred ground, and led to later ritualistic use of the enclosure. It could have been a focus for activities such as the feasts suggested to have occurred at causewayed camps in southern Britain⁶.

If we suppose that events such as markets, feasts and the kraaling of cattle took place at certain set times of the year, the Coupland enclosure is perhaps not so far removed from Stonehenge after all, where seasonal astronomical events were clearly of great importance. Although there is no evidence at the Coupland site for a circle monument inside the henge (wood, stone or otherwise), the architectural antecedents for the henge tradition — outer bank and internal ditch — may have their roots in this and other, as yet undiscovered, sites. □

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Time to get excited by GABA

Christopher S. Colwell

Much of the inhibitory synaptic transmission that occurs in the brain is thought to be mediated by γ -aminobutyric acid (GABA). Synaptically released GABA activates GABA_A receptors, leading to the opening of chloride-permeable ion channels. The resulting hyperpolarization (as well as the underlying increase in conductance) inhibits the electrical activity of the neuron, making it less responsive to excitatory input. This model is so well accepted as the basis for fast inhibitory synaptic transmission that the mere presence of the enzyme that is responsible for making GABA is usually enough to label a particular pathway as being inhibitory.

But there is now evidence that GABA can also act as an excitatory transmitter. On page 598 of this issue, Wagner and co-workers¹ report that mature neurons in the suprachiasmatic nucleus (SCN) of the hypothalamus can be excited by GABA, through a GABA_A-dependent mechanism. Cells in the SCN are responsible for the generation of circadian rhythms in mammals, undergoing spontaneous changes in their electrical properties between day and night. Interestingly, the effect of GABA also seems to show diurnal variations — the excitatory response is seen only during the day. So, at least for SCN neurons, the time of day determines whether GABA acts as an inhibitory or an excitatory transmitter.

What mechanisms are responsible for

this daily change in the action of GABA? Because GABA_A-gated channels are selectively permeable to chloride ions, the GABA equilibrium potential is largely determined by the chloride equilibrium potential. The GABA equilibrium potential is the membrane potential at which no net current will flow, and the membrane will tend to move to this potential after the application of GABA. In the model proposed by Wagner *et al.*¹, SCN cells show a daily rhythm in the GABA equilibrium potential such that, during the day, it is several millivolts positive relative to

the resting membrane potential (Fig. 1). Accordingly, during the day, when GABA opens the chloride ion channel, the membrane potential becomes more positive and action potentials are generated.

The opposite happens during the night, because the GABA equilibrium potential is negative relative to the resting membrane potential. So GABA acts as an inhibitory neurotransmitter, decreasing the number of action potentials that are generated. The most likely explanation for these fluctuations in the GABA equilibrium potential is, perhaps, a diurnal change in the intracellular concentration of chloride. Unfortunately, it has proven technically difficult to measure the GABA equilibrium potential of SCN cells without altering the internal chloride concentration.

Although it is not yet clear how common the action of GABA as an excitatory transmitter may be, there is a precedent for time-dependent changes in its effects. For example, in the developing nervous system, it is well established that GABA can excite cells². The mechanism seems to be an elevation in the levels of intracellular chloride in immature neurons. So activation of GABA_A receptors would elicit a depolarizing current, and GABA could function as an excitatory transmitter. But, in these cases, the excitatory effect of GABA becomes inhibitory as the neurons mature.

In another example, large amounts of exogenous GABA or the high-frequency activation of inhibitory synapses produce a biphasic response in the brain of mature animals³: initial hyperpolarization is followed by an overshoot to depolarizing and action-potential-generating potentials. So there seem to be a growing number of situations in which it is appropriate to think of GABA as being a transmitter that can be excitatory or inhibitory, depending on the physiological conditions.

The observation by Wagner *et al.*¹ — that basic properties of synaptic transmission

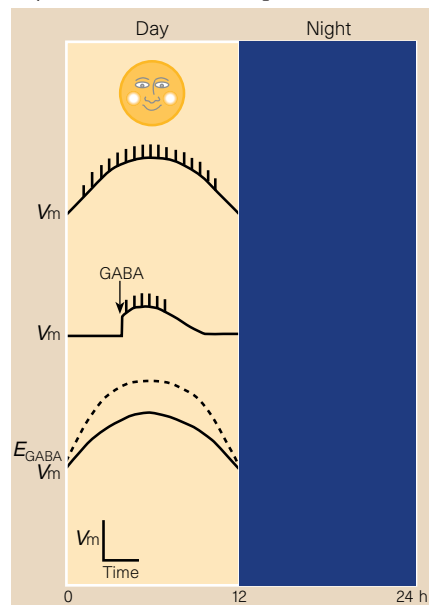


Figure 1 The neurotransmitter γ -aminobutyric acid (GABA) can be either excitatory or inhibitory in cells of the suprachiasmatic nucleus (SCN). These cells are responsible for the generation of circadian rhythms in mammals, and undergo spontaneous changes in their electrical properties (V_m) between day and night. Wagner *et al.*¹ now report that the effect of GABA on these cells also varies with the time of day, because GABA produces an excitatory response during the day, but not during the night. The underlying mechanism seems to be a daily shift in the GABA equilibrium potential (E_{GABA}) from being depolarized relative to the resting membrane potential during the day, to being relatively hyperpolarized during the night. Daily changes in intracellular chloride concentration are thought to drive this switch from GABA-mediated excitation to inhibition.

could vary with the time of day — is fundamentally important. Most organisms, including humans, show daily rhythms in their physiology and behaviour. Although most of these rhythms are seen at the level of the output of a physiological system, the new study shows that even the most basic property of cell–cell communication in the nervous system (that is, whether the release of transmitter excites or inhibits the postsynaptic cell) can also vary with the circadian cycle. Add to this observations that the properties of a cellular membrane, its second-messenger systems and its transcriptional/translational machinery can all be rhythmically regulated^{4,5}, and the emerging view is that even the most basic cellular and molecular properties of a cell can be subject to circadian regulation.

What does the work by Wagner *et al.*¹ mean for our understanding of the circadian rhythms that are generated by cells in the SCN? Most of these neurons send out processes containing GABA, which form synapses with other cells in the SCN. We now have to consider the possibility that these cells switch from being excitatory to inhibitory interneurons, depending on the time of day. So is this switch responsible for generating the daily rhythm in spontaneous neural activity that is characteristic of SCN

cells? Probably not, as there is evidence to suggest that this rhythm is generated by intracellular processes that do not depend on synaptic communication⁶. But the switch in sign may boost the amplitude of the neural rhythm, and be involved in driving the outputs from the SCN.

Agonists towards GABA can have an impact on the circadian system⁷, so there is every reason to think that understanding the physiological role of GABA-mediated synaptic transmission in the SCN will help to explain circadian phenomena in mammals. The findings of Wagner *et al.* also force us to re-evaluate our thinking about GABA as a signalling molecule — they raise the possibility that under certain physiological conditions, or at certain times, GABA can act as an excitatory transmitter. Certainly, for SCN cells, there are times to be excited by GABA. □
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Biosensors

Switching channels makes sense

Anthony P. F. Turner

The Atlas moth can follow a thinly laid trail of single molecules. Can we mimic it? Building a device to do that, and so pass the sternest test of sensitivity, is one of the ultimate goals of the biosensor technologist. Pursuit of this prize is likely to lead to considerable commercial benefit from the production of highly sensitive diagnostics, and exciting possibilities for computers that use biochemicals in place of conventional solid-state electronics. On page 580 of this issue¹ Cornell *et al.* describe an important step forward — a stable lipid membrane that can act as an ion gate, opened or closed by the binding of single molecules.

Over the past decade, biosensors (sensors incorporating a biological or biologically derived sensing element²) have achieved considerable commercial success, with current worldwide sales of over half a billion US dollars a year. The field has been dominated by enzyme-based biosensors, and in particular by disposable enzyme electrodes for home blood-glucose measurement³. Immunodiagnosics and other affinity systems have so far been relatively unaffected by this emerging technology, with consumer products focusing on immunochromatographic approaches such as the new test

strips for pregnancy and fertility.

Biosensor technology has had a smaller but significant impact on affinity assays performed in the laboratory, especially in the pharmaceutical industry. Several companies sell real-time bioaffinity assays based on measuring the change in refractive index resulting from antibody–antigen interactions at a sensing surface. The best known of these exploit surface plasmon resonance (oscillations of electrons in a thin metal layer) at an optical interface to measure affinity reactions occurring in a thin polymer matrix coating⁴. Many companies are working to miniaturize this and related technologies, in an attempt to produce a handheld immunosensor that might be similar in both cost and size to the pocket-sized glucose biosensors already on the market. Such instruments could be used for home testing or for non-medical applications such as environmental or food monitoring. Target analytes include toxins, allergens, disease markers, hormones, bacteria, viruses, antibodies, DNA, drugs and pesticides.

The idea of mimicking nature by harnessing an affinity reaction to open a gate or switch in a membrane is not new. Various ion-channel sensors were described in the late 1980s, and by 1989 there remained three



100 YEARS AGO

In connection with the recent correspondence in these columns on luminous phenomena observed on mountains, it is interesting to direct attention to a very remarkable series of observations of electrical storms on Pike's Peak, Colorado, contained in vol. xxii. of the *Annals of the Astronomical Observatory of Harvard College*, and described in *NATURE*... Luminous jets appeared very often along the telegraph wires for the length of an eighth of a mile, and the anemometer cups looked like revolving balls of fire. Upon touching the anemometer under these conditions, an observer found "his hands instantly become aflame. On raising them and spreading his fingers, each of them became tipped with one or more cones of light nearly three inches in length." From *Nature* 3 June 1897.

50 YEARS AGO

Prof. J. Kaplan proposes the name 'active oxygen' for certain luminous phenomena observed by him "just as the name active nitrogen was given to similar phenomena in nitrogen". I write to point out that this is not historically correct. ... The chemical activity referred to was the combination with metals such as sodium and mercury, to form nitrides, and with organic materials to form cyanogen compounds. Striking luminous phenomena often accompany these chemical actions, but it is wrong to regard these effects as the essence of the matter. As a matter of fact, the number of nitrogen molecules which emit a photon of afterglow light is very small compared with the number which become chemically active. For this reason, I do not think that the afterglow should be regarded as the essential phenomenon of active nitrogen, and I do not think that in the present state of knowledge the term 'active oxygen' should be applied by analogy, when only luminous phenomena are so far known to be involved. This would only cause confusion. From *Nature* 7 June 1947.

Many more abstracts like these can be found in *A Bedside Nature: Genius and Eccentricity in Science, 1869–1953*, a 266-page book edited by Walter Gratzer. Contact David Plant (e-mail: subscriptions@nature.com).

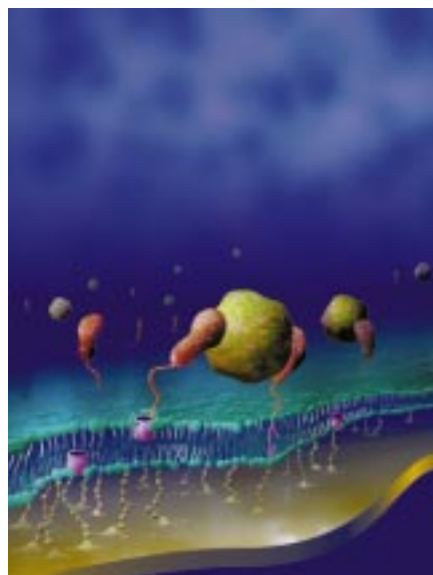


Figure 1 An artist's impression of the new ion-channel biosensor in operation. A molecule of the desired analyte (yellow) is bound to two tethered antibodies. One of them is fixed, the other tethered to a mobile gramicidin molecule (pink cylinder), which has thus been pulled away from its fixed lower partner, severing the ion channel and interrupting current to the gold electrode below. A single (and highly specific) molecular interaction results in a detectable change in electrical conductance.

technical problems⁵ in achieving a practical device: the fabrication of stable lipid membranes; the incorporation of a receptor into these structures; and the marriage of the modified membrane to a transducer, to convert the chemical signal into an electrical one. Despite excellent work since then^{6,7}, these problems have remained largely unresolved.

But Cornell *et al.*¹ have gone some way towards making a practical device (Fig. 1). To improve stability, they include membrane-spanning lipids (as in thermophilic bacteria), and the membrane is tethered to an underlying gold electrode. Each ion channel is formed of two linked gramicidin molecules; a fixed one, tethered to the electrode below, and a mobile one in the upper layer of the membrane, linked to an antibody fragment specific to the chosen analyte. The availability of mobile gramicidin to form channels is modulated by a second tethered antibody fragment (see Fig. 1). Polar molecules between the membrane and the electrode leave a space that acts as a reservoir into which cations can flow when the channel is opened, and the electrode acts as the transducer.

An advantage of such gated devices is the very high flux (up to a million ions per second) that occurs as the result of a single molecular interaction. This type of amplification lies at the heart of biological systems, and has yielded artificial sensors capable of detecting sub-picomolar concentrations within ten minutes.

It will be possible to incorporate ion-channel sensors in miniature sensor arrays, designed to respond to a wide range of analytes. Indeed, as the membrane area decreases, the membrane leakage conductance decreases, whereas the conductance per channel remains constant. So with small electrodes (<30 μm across) it should be possible to resolve individual channels. In principle, a wide range of receptor molecules could be incorporated into such a system. Antibody libraries or combinations of synthetic receptors could provide a diverse range of potential binding partners, which could be selected empirically by exposure to the target analyte. A certain amount of redundancy is usual in living systems, and high-density arrays could exploit duplication and accommodate crossreactivity by using neural-network approaches to produce the bioelectronic nose or tongue. Such arrays should be able to cope with the most stringent of demands, including chiral discrimination — the subject of a paper by Bodenhöfer *et al.* on page 577 of this issue⁸, in which right- and left-handed enantiomers of a chiral receptor are coated onto sensor arrays that respond to the mass or optical thickness of the surface layer.

On a more speculative note, the creation of a stable bioamplifier has implications beyond the field of analytical chemistry.

Microbial genetics

Hypermutation under stress

Bryn A. Bridges

When a population of microorganisms is unable to grow because nutrients are exhausted or cannot be used, it could make good sense for individuals in that population to experiment with their genomes to try to overcome the deficiency. One approach would be for a subpopulation to start mutating vigorously at random (hypermutation), in the hope that a mutation might arise that would enable growth to begin again. An alternative strategy would be to mutate only those genes that might possibly be effective if mutated (directed mutation), an idea that was raised by John Cairns and his colleagues in much-cited work published in 1988¹. Two new papers, one from Pat Foster² and the other from Susan Rosenberg and her group³, turn the spotlight firmly on the first of these strategies.

Whereas some evolutionary biologists found the idea of directed mutation disturbing because of its echoes of Lamarckism, molecular biologists displayed their customary ingenuity in proposing ways in which it might be achieved without threatening conventional dogma (for review see ref. 4). It is now clear that mutations may arise at an unexpectedly high rate during nutritional stress, and the

Single-molecule modulation of a gated event is a cornerstone of biomolecular electronics, and so this new device brings us a small step closer to a new generation of organic machines. The immediate commercial impact of the work by Cornell *et al.*¹, however, is likely to be restricted to a new range of simple immunodiagnoses. If their results translate to superior practical devices, that alone could have an enormous effect on users and providers of affinity assays for medicine, the environment, food, process monitoring, security and defence. And one can envisage numerous applications as a research tool, ranging from screening of pharmaceuticals to *in vitro* studies of cellular activation. □

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mechanisms concerned are equally interesting whether they involve hypermutation or directed mutation (or even both). Although there have been many attempts to prove or disprove the hypothesis of directed mutation, none has been totally convincing.

The most studied system is the FC40 strain of the bacterium *Escherichia coli*, in which the chromosomal *lac* operon is deleted and in which there is an F' episome carrying a *lacI* gene with a +1 frameshift mutation⁵. Being *lac*⁻, the bacteria cannot use lactose. But on agar plates containing lactose as the sole carbon source, mutant *lac*⁺ colonies arise at a high rate provided the bacteria are recombination-proficient. The F' episome is a sexual (conjugational) plasmid with some homology to the bacterial chromosome, and conjugational potency is necessary for the high mutation rate.

A few years ago, Foster⁶ found that although *lac*⁺ mutations accumulated during selection for lactose utilization, unselected mutations at the chromosomal *rpoB* locus did not accumulate in the *lac*⁻ population. Now, however, the same author² has shown that unselected tetracycline-resistance mutations arise at roughly the same

rate as the *lac*⁺ mutations under these conditions — that is, the mutagenic process is not directed solely to the *lac* gene. Both the tetracycline-resistance and the *lac*⁺ mutations are on the F' episome and consist largely of single base deletions in runs of G–C base pairs. Indirect evidence is consistent with mutations in both genes occurring in a small subpopulation of highly mutating cells in which DNA is actively recombining.

Rosenberg and colleagues have pursued a subtly different line in looking for unselected mutations. Among the *lac*⁺ mutants that arise during selection for lactose utilization, they now demonstrate³ the presence of a high frequency of tetracycline-resistance mutations on another plasmid, and of mutations in other episomal and chromosomal genes. Like Foster, they argue that a recombinational mechanism is responsible and give persuasive evidence that only a small proportion of the population is mutating at the high rate.

Unlike Foster, however, they did not see any unselected mutations in the population that remained *lac*[−], a result that at first sight appears to conflict with her observation². This apparent discrepancy is probably due to differences in experimental method. Foster examined all the *lac*[−] cells on her mutant plates, some 10⁸ per plate, and found that the rate of mutation to tetracycline resistance was about 2.4×10^{-7} per cell per day under conditions of selection for lactose use (80 per cent of the rate of mutation to *lac*⁺). Rosenberg's group picked off individual *lac*[−] colonies and screened fewer than 10⁴ for tetracycline-resistant mutants, finding none. Even allowing for the fact that the plasmid has a relatively high copy number, the sensitivity for detection of tetracycline-resistance mutants was probably some two orders of magnitude less than that of Foster.

A similarly reduced sensitivity applies to Rosenberg and colleagues' search for episomal mutants at other loci. The fact that they were able to detect unselected mutations only among the *lac*⁺ colonies is probably because only a minority of the population is hypermutating, and the *lac*⁺ mutants are exclusively drawn from this subpopulation. So the two sets of results^{2,3} are not inconsistent and together show the generality of hypermutation under lactose selection. In the FC40 system, therefore, mutation is not directed. Mutations accumulate not only in the gene being selected for but also in other genes on the episome, on the chromosome and on another plasmid, although not all loci appear to respond to the same extent.

There is, however, a further discrepancy between these new results^{2,3} and Foster's earlier observation⁶ that rifampicin-resistant mutations in the *rpoB* gene do not accumulate in the FC40 strain during lactose selection. It could be that the *rpoB* gene may be one of those that does not respond to hypermutation, but the simplest

explanation is that the FC40 type of hypermutability does not include the generation of many base-pair-substitution mutations such as those that confer rifampicin resistance. The mutations that show hypermutability in the experiments of Foster and Rosenberg's group are either −1 frameshifts or uncharacterized mutations that inactivate gene function, which could include frameshifts and similar small deletions.

Random hypermutation in specific genes is a tactic that has been evolved by numerous pathogenic microorganisms in doing battle with their host's defences (see ref. 7 for review). In contrast, the latest results with the FC40 system seem to indicate a specific type of hypermutation in random genes. Specific mutational events are also characteristic of mutations arising during nutritional stress in bacteria that do not carry F' episomes. In particular, G to T mutations are thought to arise as a result of miscoding events opposite DNA lesions that accumulate during starvation^{8,9}. DNA damage may also be important in generating −1 frameshifts. Rosenberg's group has postulated¹⁰ that double-strand DNA breaks are generated during stress and constitute the trigger for recombinational processes that result in −1 frameshift muta-

tions in the FC40 system. Alternatively, oxidative damage may be involved here too. Along with G to T mutations, −1 frameshifts (G deletions) were the predominant events when single-stranded phage DNA was exposed to singlet oxygen¹¹.

We shall see. In all, it would perhaps be wise to reserve judgement on whether the hypermutation observed in FC40 has evolved as a means of overcoming nutritional stress (and thus whether it might have some evolutionary or ecological significance) or whether it is essentially a pathological response to damage accruing during the stress. □

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Electromagnetism

The secret life of the dipole

Jeeva S. Anandan

We are familiar with the acceleration of charged particles, such as protons and electrons, by uniform electromagnetic fields. But neutral particles can be accelerated by uniform fields too, if they possess a magnetic dipole^{1,2}. Some consequences and the possibility of observing this force are the focus of a new study³.

The force on a magnetic dipole in an electromagnetic field has been studied for more than 100 years. The expression found in textbooks depends on the spatial gradient of the applied magnetic field ($\mathbf{F} = \nabla(\boldsymbol{\mu} \cdot \mathbf{B})$, where $\boldsymbol{\mu}$ is the magnetic moment and $\mathbf{B}$ is the

magnetic field). If we imagine the magnetic dipole to be an infinitesimal current loop, then $\mathbf{F}$ may be understood as the net force resulting from the different Lorentz forces acting on different parts of the loop.

So it came as a surprise to me to find, eight years ago^{1,2}, that there is another force: $\mathbf{f} = \tau(\mathbf{B}' \times \boldsymbol{\mu}) \times \mathbf{E}$, where $\mathbf{E}$ is the electric field, $\mathbf{B}' = \mathbf{B} - \mathbf{v} \times \mathbf{E}$ is the magnetic field in the rest frame of a dipole that is moving with velocity $\mathbf{v}$ relative to the laboratory, and τ is the ratio of the magnetic moment to the spin $\mathbf{S}$ of the dipole (using units in which the velocity of light is 1). This force exists even when the fields do not

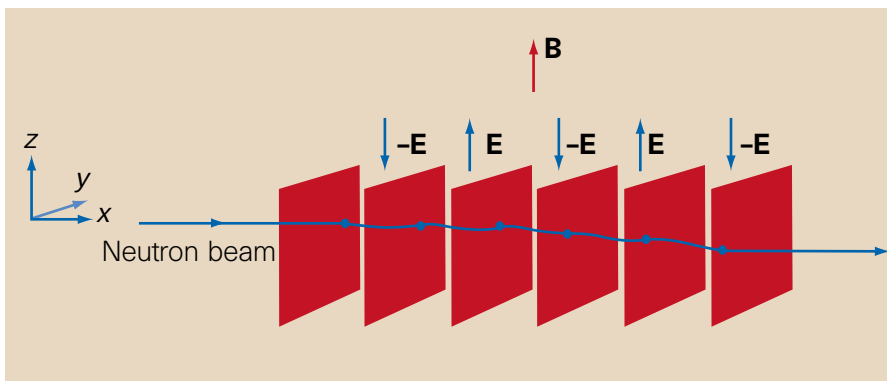


Figure 1 A neutron beam moving through a sequence of cells containing alternating electric fields. This configuration has been chosen to make the acceleration accumulate. The blue curved line represents the trajectory of a neutron wave packet if its initial spin was in the y direction (see Fig. 2).

vary in position, so it cannot be easily obtained from the above intuitive picture. It is surprising also because of its dependence on the electric field: as the current loop representing the dipole may have no charge, it might be expected not to couple to an electric field. Also, this force is doubly nonlinear; it does not reverse in direction if either the electromagnetic field strength or the magnetic moment is reversed. I know no other force that is nonlinear in the field strength (as opposed to the potential). But after four separate derivations^{1,2}, in quantum and classical physics, I was convinced that it does exist.

A key to understanding the new force is that for this dipole the kinetic momentum (classically $m\mathbf{v}$) differs from the canonical momentum $\mathbf{p}$. The canonical momentum is associated with the wave nature of the particle and is conserved if there is translational symmetry in space, as when the dipole interacts with a uniform electromagnetic field. In fact, $m\mathbf{v} = \mathbf{p} - \mu \times \mathbf{E}$. The rate of change of $m\mathbf{v}$ is the total force, by Newton's second law. The time derivative of $\mathbf{p}$ is $\mathbf{F}$, and the time derivative of the second term, called the *hidden* momentum, which is due to relativistic effects, is $\mathbf{f}$ (assuming that $\mathbf{E}$ is time independent).

For the neutron, it turns out that when $B = 1$ tesla and $E = 10^7$ V m⁻¹, which can be realistically achieved in the laboratory, the acceleration caused by the new force is of the order of 12 cm s⁻². On the face of it, this acceleration is large enough to be seen easily. But the neutron's spin (and therefore its magnetic moment, which is antiparallel to the spin) precesses rapidly about $\mathbf{B}'$ due to the torque it experiences. With a precession frequency here of 1.8×10^8 radians s⁻¹, $\mathbf{f}$ averages to zero very quickly. That is why it has not already been observed in the large number of experiments in which a dipole interacts with an electromagnetic field. I suggested that to make the effect of the force accumulate, instead of cancel out, $\mathbf{B}$ could be kept con-

stant while $\mathbf{E}$ alternates in space^{1,2}.

Now Wagh and Rakhecha³ have studied this proposal in detail. A neutron is assumed to pass through a sequence of cells each having length l and containing transverse uniform electric fields $-\mathbf{E}, \mathbf{E}, -\mathbf{E}, \mathbf{E} \dots$ such that $\mathbf{E}$ is parallel to a uniform magnetic field $\mathbf{B}$ (Fig. 1). The length l is chosen so that the time taken for the neutron to travel each cell is half the Larmor period — that is, the time for its spin to rotate once around the magnetic field $\mathbf{B}'$.

Given the geometry of Fig. 1, if the spin $\mathbf{S}$ starts by pointing in the y direction then it will precess around $\mathbf{B}'$ — approximately rotating in the x - y plane, as $\mathbf{B}'$ is close to $\mathbf{B}$. As the neutron traverses the first cell, its spin and magnetic moment rotate by 180°, so S_x is always negative, whereas S_y is positive and negative for equal durations inside this cell. In the next cell, S_x is positive, but owing to the reversal of the electric field, f_x is positive again. Hence, the velocity steadily increases in the x direction, whereas in the y direction it fluctuates as shown in Fig. 2.

The cumulative effect could be detected by allowing the accelerated beam of neutrons to interfere with an unaccelerated beam. In neutron interferometry, the fringe contrast is greatest when both beams are in the same spin state, so Wagh and Rakhecha suggest letting the neutron go through another series of cells, to restore its original spin state.

But an unambiguous way of observing $\mathbf{f}$ is from the deviation of the neutron beam in the y direction⁴. As shown in Fig. 2, this component would be zero in the absence of the new force $\mathbf{f}$ because of the translational symmetry in this direction. In the presence of it, v_y is negative in each of the cells, and therefore the beam deviates increasingly towards the negative y direction as it passes through the cells. The detection of this deviation would be definitive evidence of $\mathbf{f}$.

The observation of $\mathbf{f}$ would provide direct evidence of the hidden momentum. If that is the only objective, it can be achieved more simply by passing the neutron into a region of uniform $\mathbf{E}$ -field, with no $\mathbf{B}$, such as the space between two capacitor plates, and measuring its velocity change due to the change in the hidden momentum $\mu \times \mathbf{E}$. But the earlier proposal has the advantage that it would detect $\mathbf{f}$, which would be interesting also because $\mathbf{f}$ is the analogue of the nonlinear terms in the gauge-field forces that govern weak and strong interactions, which cannot be seen in the same manner because they are short range. □

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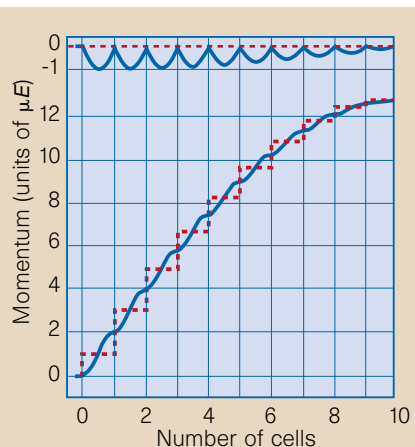


Figure 2 The x and y components of the mean kinetic momentum of the neutrons in Fig. 1, without (dotted lines) and with (solid lines) the additional force $\mathbf{f}$. (Adapted from ref. 3.)

Daedalus

Flower power

Plants have a rich, even a rococo, chemistry. They elaborate many strange and complex substances — terpenes, alkaloids, phenols, and so on, widely exploited in herbal medicine. Yet this medicine rests on uneasy foundations. For all these plant metabolites have but one purpose — to discourage or kill anything that tries to eat the plant.

Fortunately, most plant metabolites are targeted, not at us, but at insects. They can even be released to counter a local insect threat. Cotton plants, for example, release a terpene vapour when attacked by armyworms. Some substance in the saliva of the insects, or generated by its attack, triggers the release of the vapour.

Daedalus sees this as a splendid new source of herbal fuel. Such fuels are being rapidly developed — witness 'biodiesel' oilseed fuel, and the recent Indian herbal fuel fraud. Plant terpenes, the constituents of turpentine, are an excellent hydrocarbon fuel. They are metabolically 'cheap', too — some tropical shrubs and trees release such copious terpene vapours that the scent carries for kilometres.

There's a claim that it can be a chemical signal to other plants. So DREADCO biochemists are placing various insects on the leaves of terpene-releasing plants, to study the detailed chemistry of their mastication. They hope to identify the trigger compound that tells a plant that it is under insect attack, and must switch all its resources to terpene synthesis.

Once identified and synthesized, the terpene trigger will open the way to a new fuel technology. A plantation of shrubs or trees — pine, perhaps, or eucalyptus — will be enclosed in a huge plastic-film greenhouse. The terpene trigger will be pumped in, and every plant will start pouring out terpene vapour as if its life depended on it. The greenhouse air will be continuously extracted, and cooled or passed over absorbents to collect the terpenes, which will be re-formed into motor fuel. The level of terpene trigger will need to be carefully regulated. Just enough must be supplied to divert most of the plants' metabolic effort into terpene production, without sapping their vitality.

This elegant technology has all the ecological virtues. Unlike other biofuel schemes, it needs no labour-intensive planting and harvesting. The fuel ultimately comes from the carbon dioxide of the atmosphere; when burnt, it returns without augmenting it. And the plantations will not need expensive insecticidal spraying.

David Jones

Obituary

Norman Pirie (1907–97)

Biochemist who characterized plant viruses and promoted leaf protein as food

Norman Wingate Pirie, invariably called Bill, died on 29 March aged 89. He was a biochemist trained in the pre-war Cambridge school of Sir Gowland Hopkins, but he had the mark of a polymath. His penetrating mind encompassed an encyclopaedic knowledge of scientific discovery and etymology, and his forceful writings on the origins of life, the biology of space travel, the related issues of world population, hunger and contraception, and the dangers of nuclear weapons, reflected the breadth of his scholarship, his socialist principles and his enduring concern for human welfare and world stability.

Bill was born in Stirlingshire, Scotland, the youngest child of Sir George Pirie, a noted painter. The family lived in a large house that allowed its members space to develop their own interests, and here Bill set up a boyhood laboratory. His early schooling was often disrupted, so much of his learning came from books and his intellectual home background, which nurtured self-reliance, independence of thought and frugality.

His practical researches had two main themes. In a highly creative pre-war period in Cambridge he studied the nature of plant viruses, then during a much longer stay at Rothamsted Experimental Station in Hertfordshire he ardently promoted extracted leaf protein as a supplementary, cheap and nutritious human food.

The virus work was a collaboration with Fred Bawden, who interested him in the problem of isolating, purifying and characterizing the viruses causing various diseases of potato. Together they obtained about a dozen viruses, or strains of viruses, in semi-crystalline or even crystalline form, including tobacco mosaic virus (TMV). Pirie demonstrated that the preparations contained small amounts of phosphorus and subsequently showed conclusively that all contained ribonucleic acid (RNA). This contradicted the early views of Wendell Stanley (a later Nobel laureate), who believed viruses consisted entirely of protein. Bawden and Pirie realized that RNA might be the infective component of viruses; but they were unable to confirm this experimentally, and it was left until 1956 for others to establish the infectivity

of RNA. Pirie took delight in demonstrating the birefringent properties of suspensions of TMV, which exhibit wonderful coloured sheens when swirled in polarized light, due to the alignment of the rod-like virus particles. With characteristic flair, he entertained the audience at a Royal Society soiree by using goldfish and sea horses to stir virus suspensions, captivating fish physiologists and plant pathologists alike.

The leaf-protein work essentially began with his move to Rothamsted in 1940, and continued almost to the week of his death. Initially he responded to a government call for research that could contribute to Britain's wartime food needs. Bill's concept was that protein extracted from leaves, such as those of cereals and broad-leaf vegetables whose fibre content means they cannot be directly digested by humans, might be used to augment a restricted supply of normal foodstuffs. Subsequently the thinking broadened, and large-scale extracted leaf protein was envisaged as a solution to fill the 'protein gap' in the diets of the increasing populations of developing nations.

With a small team, Pirie developed a variety of machines to shear the leaves, express their juices, and coagulate and filter the protein. Probably the most useful of these 'mechanical cows' were the small, simple, cheap yet robust 'village units', intended for use in poor rural communities, that needed little maintenance. Equipment was installed in villages in south India and elsewhere, and associated feeding trials established the nutritive value of the protein concentrate.

Why, then, has extracted leaf protein not had a greater impact? The reason lies not in the concept, but in the fact that during the past 50 years global agricultural production has kept pace with world population growth. The technology lies latent and may yet come into its own in the next century as the Earth's peoples continue to multiply. For the present, as Bill realized, the fact that the protein is a rich source of β -carotene, or provitamin A, may be of greater importance, because dietary deficiencies of this carotenoid cause much preventable blindness worldwide.

Formal accolades came in the form of the fellowship of the Royal Society, the invitation to give the society's Leeuwenhoek lecture, and its most distinguished award, the Copley medal. But Bill probably gained more pleasure — and wry amusement — from the Rank

Prize for Nutrition, worth £15,000, awarded to him in 1976. The cheque was immediately handed to Rothamsted with instructions to purchase an annuity on his life, the income and any accrued interest to be available to support his research in retirement. As he was then 68, the insurance company agreed an annuity of about £3,500. As each subsequent year passed, the greater Bill's pleasure became as the total approached £100,000. Rarely can a charity have made a better, or more astute, investment.

Bill's lifestyle was simple, to a degree austere. The weekly shopping was completed in five minutes on Saturday mornings, and for 57 years he lived in an estate cottage, a few minutes' walk from his laboratory where a normal working day began about 5 a.m. and continued until late each evening. This regime was maintained seven days a week, 365 days a year, except when attending conferences or advising overseas. Yes, he did work on Christmas Day, after joining Rothamsted's director and his wife and other neighbours for seasonal fare. After quickly downing two or three dry Martinis (three parts of gin and one of Martini) and various snacks, he would tender thanks and depart for the lab, usually adding the comment that he "could not understand why people spend time cooking a range of goodies when a bit of bread and cheese would be equally nutritious" — a truism from a real and rare character.

Leslie Fowden and Stan Pierpoint

Leslie Fowden and Stan Pierpoint were formerly at Rothamsted Experimental Station, Harpenden, Hertfordshire AL5 2JQ, UK.



HALIFAX COURIER LTD

Why drinking green tea could prevent cancer

Epidemiological studies suggest that the consumption of green tea may help prevent cancers in humans; also, breast and prostate cancers in animal models are reduced by green, but not black, tea¹. Here we offer a possible explanation. We have inferred (using molecular modelling) and subsequently demonstrated that one of the major ingredients of green tea inhibits urokinase, an enzyme crucial for cancer growth.

Tea is drunk in three forms: black (78%), green (20%) and oolong (2%). Green tea contains many polyphenols known as catechins, including epigallocatechin-3 gallate (EGCG), epigallocatechin (EGC) and epicatechin-3 gallate (ECG). The brewing of black tea oxidizes

the catechins, destroying any beneficial effects¹. Several mechanisms of anticancer activity of catechins have been postulated, but none seems universal for all cancers¹⁻³.

Human cancers need proteolytic enzymes to invade cells and form metastases. One of these enzymes is urokinase (uPA). Inhibition of uPA can decrease tumour size or even cause complete remission of cancers in mice^{4,5}. The known uPA inhibitors are unlikely to be used in anti-cancer therapy because of their weak inhibitory activity or high toxicity.

We have searched for new uPA inhibitors by computer modelling using the active site of uPA as a template. Coordinates of human uPA were kindly provided by C. Phillips⁶; National Cancer Institute,

MayBridge, and Merck 3D databases of 190,000 compounds were used to select inhibitors by Biosym LUDI and DOCKING programs. In these calculations, the relative position of inhibitor and receptor (uPA) with the minimum potential energy represents the most probable way of binding. Polyphenols, among other compounds, showed good inhibitory potential. One of them, EGCG (a component of green tea), binds to uPA, blocking His 57 and Ser 195 of the uPA catalytic triad and extending towards Arg 35 from a positively charged loop of uPA (Fig. 1a). Such localization of EGCG would interfere with the ability of uPA to recognize its substrates and inhibit enzyme activity⁶.

We have verified our computer calculations using an amidolytic assay of uPA activity in the presence of different concentrations of EGCG. The uPA activity was quantified spectroscopically using Spectrozyme, which releases a chromogen on specific cleavage by uPA. EGCG from two different suppliers showed almost identical rates of uPA inhibition.

We have compared the ability of EGCG to inhibit uPA with that of a well-known inhibitor, amiloride, and a control sample where no inhibitors were used (Fig. 1b). EGCG is a weaker inhibitor than amiloride, but can be consumed in much higher doses without any toxicological effects. Amiloride is administered in a maximum dose of 20 mg per day, whereas a single cup of tea contains 150 mg EGCG, and some tea lovers consume up to 10 cups a day¹.

Such high levels of a uPA inhibitor are likely to have a physiological effect and could reduce incidence of cancer in humans or the size of cancers already formed. Theoretically, EGCG might inhibit cancer formation in many different ways; however, we postulate that the well-known anticancer activity of green tea is driven by inhibition of uPA, one of the most frequently overexpressed enzymes in human cancers.

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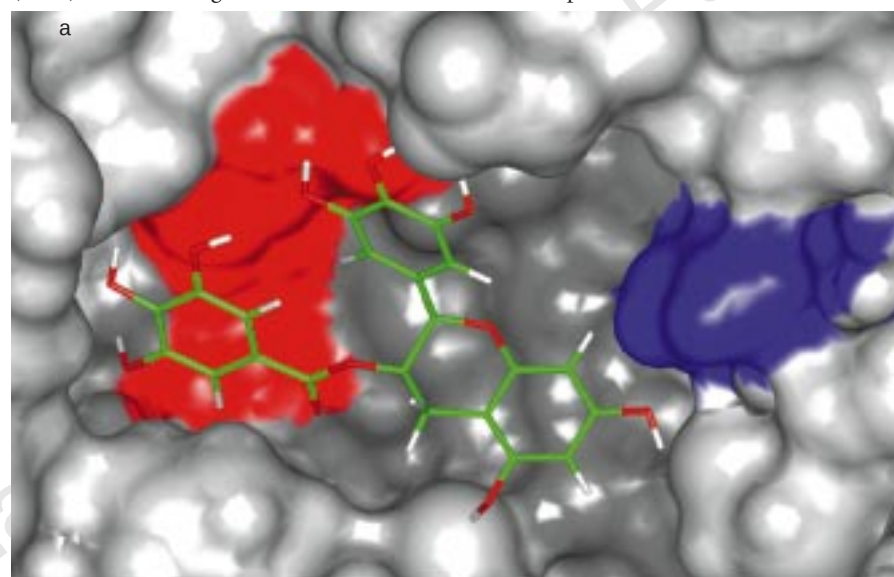
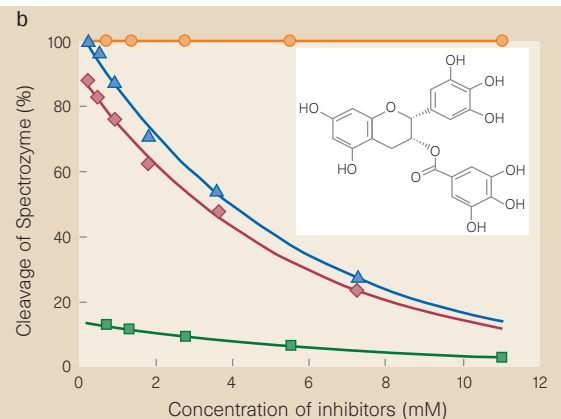


Figure 1a, Connolly surface of uPA showing the catalytic triad His 57, Asp 102 and Ser 195 (red) at the bottom, and Arg 35, Arg A37 (blue) at the brim of the cavity. EGCG, well fitted into this cavity, is shown as a 'stick model' in green (C), red (O) and white (H). The calculated energy of intermolecular interaction between EGCG and uPA is $-116.81 \text{ kcal mol}^{-1}$; LUDI score, 498; calculated K_i , $1.04 \times 10^{-5} \text{ M}$. **b**, Cleavage of Spectrozyme by uPA in the presence of EGCG (inset) from Sigma (blue triangles), and from MayBridge, UK (purple diamonds); amiloride (green squares), and control sample (orange circles). Experimental mixtures (50 mM Tris with 0.01% Tween 80, 0.01% PEG 8000 buffer; pH 8.8) were incubated with $1 \mu\text{g}$ of uPA and decreasing amounts of inhibitor for 15 min. $100 \mu\text{l}$ of this mixture was incubated in a 96-well microplate with $50 \mu\text{l}$ (2.5 mM) Spectrozyme (carbobenzyl-L-(γ)-Glu(α -tBuO)-Gly-Arg-p-nitroanilide, $2\text{C}_2\text{H}_5\text{OH}$ from American Diagnostica Inc., Greenwich, Connecticut), for 10 min. Absorbance, which is inversely proportional to the uPA inhibitory activity⁷, was measured at 405 nm on a microplate reader.



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Domatia mediate plant–arthropod mutualism

Leaf domatia are small hair-tufts or pockets on the lower surface of leaves, and are exceedingly common among perennial angiosperms, having been reported in 277 plant families and nearly 2,000 species¹. Domatia seem to provide refuges for predatory arthropods. Here we show that cotton plants with experimentally added leaf domatia host larger populations of predatory arthropods and smaller populations of herbivorous mites than control plants. Total fruit production was increased by 30 per cent in plants with domatia—the first demonstration that plants benefit from their presence.

Leaf domatia are ubiquitously inhabited by primarily predatory (rather than herbivorous) arthropods (Fig. 1), leading to the conclusion that there is mutualism between arthropods and plants^{2,3}. Previous studies have shown that blockage or addition of domatia results in positive associations between domatia and populations of predatory arthropods^{2,4,5}, but to comprise true mutualism the fitness of the plant must increase as a result of a reduction in the populations of herbivorous arthropods or other plant parasites.

We simulated the presence of domatia on cotton plants (*Gossypium hirsutum*), close relatives of which have naturally occurring leaf domatia⁶, by attaching a small tuft of cotton fibres to the abaxial surface of leaves where the veins meet. Among the most severe pests in the San Joaquin Valley, California, are herbivorous spider mites⁷. They became established early in the season on the cotyledons of experimental plants grown near Chowchilla, but we found that there were consistently fewer spider mites on plants with domatia (Fig. 2a). Predatory arthropods were found in the simulated domatia, indicating that suppression of spider mite populations might be related to the presence of domatia.

The western flower thrips, *Frankliniella occidentalis*, an opportunistic predator of mites^{8,9}, was more abundant on plants with artificial domatia (Fig. 2b), and thrips were usually found in domatia. Other predators of spider mites, the big-eyed bug (*Geocoris* spp.) and the minute pirate bug (*Orius tristicolor*), were five times more abundant on domatia-bearing plants (Fig. 2c), and their eggs and nymphs were found almost exclusively within domatia.

We found that the addition of leaf domatia early in the season improved the overall yield (number of bolls) of cotton by almost 30 per cent (Fig. 2d; $t = -2.63$, d.f. = 103.1, $P = 0.01$). This increase in yield was found



Figure 1 Adult big-eyed bug (*Geocoris* sp.), a voracious predator of herbivorous spider mites.

in a species known to tolerate high levels of herbivory without reductions in yield¹⁰.

Later in the growing season we ran an identical experiment in Davis, California. The density of spider mites was below 'pest status' on six sampling dates (mean $\pm$ s.e.m., 4.3 ± 0.43 mites per leaf). Populations of thrips were also low (1.5 ± 0.11 thrips per leaf) and were only affected by domatia in

the early part of the season. Because we initiated this experiment in the mid-season, arthropods that colonized early in the season were less affected by domatia than in the previous experiment.

Arthropods whose populations peak later in the season, such as big-eyed bugs and minute pirate bugs, showed strong responses to leaf domatia in Davis plots (big-eyed bugs, 0.5 ± 0.05 per leaf with domatia and 0.1 ± 0.02 without, $F_{1,117} = 43.20$, $P < 0.001$; minute pirate bugs, 0.6 ± 0.06 per leaf with domatia, 0.2 ± 0.03 without, $F_{1,117} = 32.52$, $P < 0.001$, repeated measures ANOVA). The number of bolls produced was slightly, but not significantly, higher in plants that had leaf domatia added later in the season ($t = -1.34$, d.f. = 117, $P = 0.18$).

There appears to be a mutually beneficial effect of leaf domatia on predatory arthropods and plants. Where spider mite densities are high, domatia favour predatory arthropods and increase plant reproduction. Such benefits were not species specific as leaf domatia benefit predatory mites^{2,4,5}, thrips and bugs. Predation by thrips and bugs, mediated by leaf domatia, seems to reduce the population of herbivorous spider mites and increases plant reproduction.

Our results are promising for a natural biological control strategy as an alternative to the use of pesticides. Several crop species that are commonly damaged by mite pests

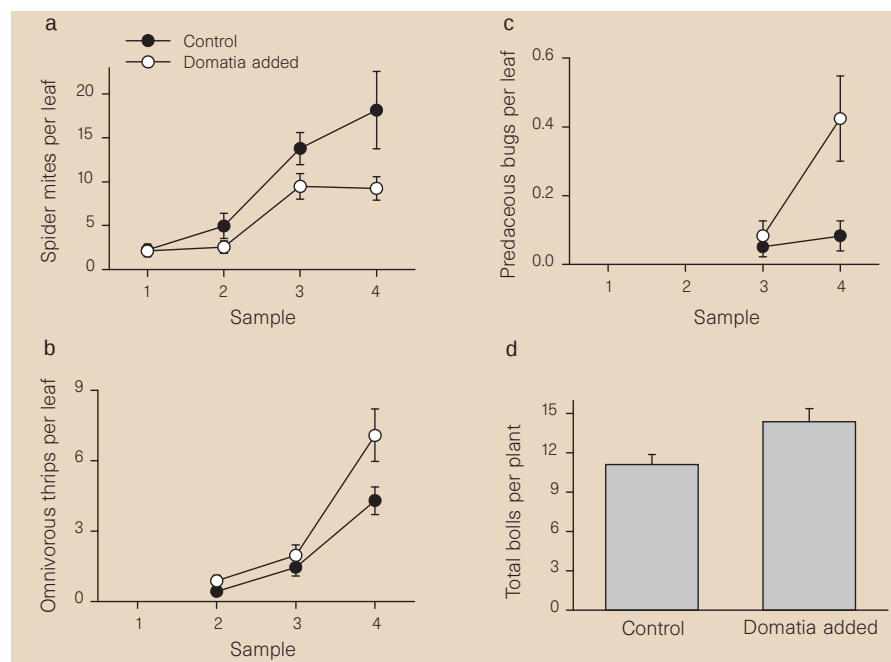


Figure 2 We attached leaf domatia to most newly expanded leaves of 60 plants during the first two months of the growing season at an organic farm near Chowchilla. **a–c**, The number of arthropods inhabiting cotton leaves with and without domatia was sampled on four occasions: 1, 10 May; 2, 24 May; 3, 7 June; 4, 21 June, on these and 60 control plants (mean $\pm$ s.e.m.). **a**, Herbivorous spider mites established early in the season and were reduced by leaf domatia ($F_{1,114} = 7.85$, $P = 0.006$). **b**, Thrips appeared in the second sample and were increased with domatia ($F_{1,114} = 6.64$, $P = 0.01$). **c**, Predaceous bugs appeared in the third sample in strongly increased numbers with domatia ($F_{1,116} = 6.46$, $P = 0.01$). As the season progressed the positive effect of domatia on predators and the negative effect of domatia on herbivores increased. **d**, Mean $\pm$ s.e.m. number of cotton bolls produced by plants with and without domatia.

have wild progenitors or relatives with leaf domatia^{1,2,11,12}. It may be possible to breed or engineer plants for better expression of leaf traits such as domatia that increase predator populations and efficacy. For plants that already have leaf domatia, these traits may mediate an important, yet poorly documented, mutualism.

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Salt enhances flavour by suppressing bitterness

Salts are used as flavouring agents in the cuisines of many cultures¹, the most commonly used being NaCl. They impart their own salty taste and enhance other flavours. The apparent ability to increase the intensity of other desirable flavours^{2,3} is puzzling as virtually all published psychophysical studies show that NaCl either suppresses or has no effect on other flavours^{3,4}. To reconcile this contradiction we have proposed⁵ that salts selectively filter flavours, such that unpleasant tastes (such as bitterness) are more suppressed than palatable ones (such as sweetness) thereby increasing the salience and/or intensity of the latter. We now present evidence to support this idea.

We used mixtures of aqueous solutions of a bitter substance, urea, which is strongly suppressed by sodium-containing compounds⁶; a sweetener, sucrose; and a salt, sodium acetate, which has a fairly mild taste⁶ and so is suitable for studying the flavour-modifying effects of sodium ions. Subjects (21 volunteers) were required to judge the extent of bitterness, sweetness and 'otherness' of all possible combinations of three concentrations of urea (0.0, 0.5, 1.0

M), four of sucrose (0.0, 0.1, 0.3, 0.5 M) and three of salt (0.0, 0.1, 0.3 M) using the method of magnitude estimation⁶. We evaluated the solutions, 12 per day (twice) over three consecutive days, in a counterbalanced order. Data were standardized and normalized⁶.

As predicted, there was a selective suppression of the taste components by sodium acetate (Fig. 1). The bitterness of urea was suppressed much more by the salt than was the sweetness of sucrose. Consequently, the sucrose–urea mixtures with added salt were relatively less bitter and more sweet than when sodium acetate was not added. Moreover, at the higher concentrations of sucrose (0.3, 0.5 M) and both concentrations of urea (0.5, 1.0 M), the absolute sweetness intensity was increased by adding either 0.1 or 0.3 M sodium acetate compared with when no sodium acetate was added (one example is shown in Fig. 1). This presumably occurred by releasing sweetness from suppression by the bitterness of urea⁷. As expected⁸, the addition of sodium acetate to sucrose in the absence of urea never had an enhancing effect on sweetness (data not shown).

Although this simple three-component aqueous system does not fully mimic the complex food systems in which salts are used, it illustrates at least one mechanism by which a salt increases both the relative and absolute intensity of palatable components of foods. This mechanism has not commonly been considered in taste mixture studies, which have tended to concentrate either on two-component mixtures, or on complex foods where interpretations are difficult.

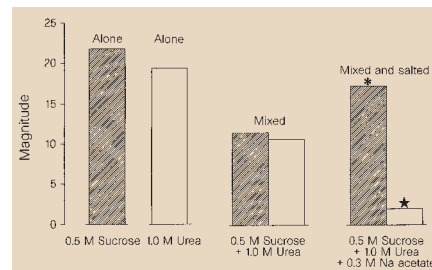


Figure 1 The normalized reported magnitude of the taste of various solution mixtures is shown. The intensity of urea and sucrose at the highest concentrations were roughly the same (left). Statistical analysis revealed that in mixtures, the highest concentrations of sucrose and urea (without sodium acetate), mutually and roughly equally suppressed their intensities (centre). When sodium acetate was added, also at the highest concentration, intensity of the bitterness greatly decreased, being suppressed by sodium ions⁶, whereas the sweetness intensity increased to levels that approximated the sweetness in pure deionized water (right). Relative to binary mixture levels, asterisk denotes increase ($P < 0.0001$) and star denotes decrease ($P < 0.0001$). These trends were evident for other concentrations tested. Detailed analyses available from the authors.

Our data show that, in addition to adding desired saltiness to food, salts potentiate flavour⁹ through the selective suppression of bitterness (and perhaps other undesirable flavours), and the release from suppression of palatable flavours such as sweetness. The desire for NaCl and other salts in foods as diverse as (often bitter) vegetables, oily foods and meats may be due in part to their ability to suppress unpleasant flavours¹⁰. This may explain why it is difficult to make low-sodium foods acceptable.

Biophysical evidence¹¹ implies that it will be extremely difficult to develop a salty-tasting sodium-free substitute for NaCl. However, the multiple sensory functions of salts in foods should be considered, as the differential flavour-suppressing effect shown here might be duplicated by non-sodium substances, such as bitterness blockers.

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Structures of mollusc shell framework proteins

Mollusc shells consist of the nacreous mother-of-pearl layer and the prismatic layer. Both layers are microlaminate composites of CaCO_3 crystals (aragonite in the nacre and calcite in the prismatic layer) and biopolymers. The main biopolymers are structural proteins, insoluble in water and methanoic acid, which determine the framework of each shell layer^{1–4} and bind soluble polyanionic proteins^{2–4} which determine the type of CaCO_3 crystal that grows^{5,6}. Here we report the sequences and structures of the framework proteins for the nacreous and prismatic layers of the pearl oyster, *Pinctada fucata*.

We ground and decalcified the nacreous layer of the mollusc shell using 50 per cent methanoic acid, and cleaved the protein by treatment with cyanogen bromide (CNBr),

before separation by Tricine-SDS-PAGE. We then western blotted the cleaved peptide onto a polyvinyl pyrrolidone difluoride filter, and obtained the amino-acid sequence, GGGFGVGLGGG (single-letter code).

We isolated two complementary DNA clones, pMSI1 and pMSI2, from a λ gt10 cDNA library constructed with poly(A)⁺ RNA from the mantle of the oyster, using a degenerate oligonucleotide probe corresponding to the amino-acid sequence FGVGLG. The pMSI1 clone encodes a glycine- and alanine-rich protein (738 amino acids) which contains the sequence GGGFGVGLGGG, matching the isolated peptide (Fig. 1a). The pMSI2 clone encodes a glycine-rich protein (334 amino acids) containing the sequences GVGLG and GVGL (Fig. 1b).

Both proteins appear to possess signal peptides, as predicted by hydropathy analysis, and have relative molecular masses of 60,000 and 31,000, respectively. We have designated these mature proteins MSI60 and MSI31. The amino-acid compositions of MSI60 and MSI31 corresponded well with those previously reported³ for the insoluble proteins from the nacreous and prismatic layers of *Pinctada fucata*, respectively.

MSI60 contains 11 poly(alanine) blocks and two alanine-rich domains, between poly(glycine)-rich regions (Fig. 1a). An X-ray diffraction study⁷ of mollusc shell proteins has shown that the insoluble matrix protein in the nacreous layer adopts an antiparallel β -sheet conformation. β -sheet domains in *Bombyx mori* cocoon silks are formed from GA/GS repeats and poly(alanine) repeats are seen in spider dragline silks⁸. The poly(alanine) blocks in spider silks are 4–8 residues long^{8,9}, whereas those in MSI60 are of 9–13 residues (Fig. 1a). Longer poly(alanine) blocks in MSI60 may form longer, densely packed crystals of β -sheets. The MSI60 protein also has 39 poly(glycine) blocks of 3–15 residues, distributed throughout the molecule (Fig. 1a). We suggest that these poly(glycine) blocks participate in the formation of crystalline β -sheets whereas the poly(aspartate) blocks in the amino- and carboxy-terminal regions may bind calcium ions. Cysteine residues located in N- and C-terminal regions could form intermolecular (and intramolecular) disulphide bonds in the nacreous sheets.

MSI31, the framework protein of the prismatic layer, has no poly(alanine) blocks, but has 10 poly(glycine) blocks of 3–5 residues in the N-terminal half of the molecule and a large acidic region including six consecutive ESEDX motifs in the C-terminal half (Fig. 1b). X-ray diffraction⁷ showed that the insoluble matrix protein in the prismatic layer is also composed of β -pleated-sheets, randomly oriented in the plane of the shell with the side chains per-

a	
MKLLVLTITLVGFSSALSFQCNYPVLGFNSQYMLGGLRLFCMPAMVYDPWACGCVSAWSAGLYGVGGG	70
GGAWGAGGAGGADGGGGGGDWEYDYDDDDDDDEWDWDDGGMGAGAGGGAGGGAGGAGAGAGAG	140
AGAGAGLGLGLGGGLGGGLGGGLGGGLGGGLGGGLGGGLGGGLGGGLGGGLGGGLGGGLGGGLGG	210
GVGGAAAAAAGGGAGRLGGAAAAAAGGAGGLGGGLGGGLGGGLGGGLGGGLGGGLGGGLGGGLGG	280
GSAAAAAAGGGGLGGVGFYGGRRGRGRGRGRRRRAAAAAAGGGGGGGGGGGGGGA	350
GAAS	420
KSSASASASASASASAGGG	490
GGGGFGGLGGGLGGGLGGGSAAAAAAGGGGGRALRRALRRQMRGGSSAAAAAAGGGWGGGM	560
GGGFGVGLGG	630
SAADVAAAAAAYGDDGADGPDGNGFGGGGNGGGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGG	700
GSGGGSGGGGNNWGNNGNNKYYDDDDCDEYGNPIRRG	738
b	
MKPFVTIASLIVLIASASADGYDDYKKYGSVGYGPGISLGGGLGGGGGIISVGGGGGLGGGLGGGLGGG	70
LVGVGGGLIGGGFGPGRVSGTINAGGGVFASGSLGGGLSPAGRGAAQGAATLSALQIASGRPRVSGVS	140
VGTGGGRAVVSATPVGFGVPGYGGYGYNYGVPSYGVGLPSYGVSLPSYGVGLGGGGGGGGYGLDLASF	210
QGSTYGNLATQINTAVVAFHMAVLLESEAESDTEVDTEMDSEEDMESEEDTESEEDTESEEDTESEE	280
DMESEEDMDESSEVDQVSMVYPNHTGVDVLCQVRLQLEFPALALVSVVLE	334

Figure 1a, Deduced amino-acid sequence (single-letter code) of pMSI1 (DDBL/EMBL/Genbank accession number D86074). The putative signal peptide is indicated by a dotted line, and the sequence detected by Edman degradation of CNBr-cleaved peptides is underlined. Poly(alanine) blocks are highlighted. **b**, Deduced amino-acid sequence of pMSI2 (accession number D86073). The putative signal peptide is again shown by a dotted line, and sequences whose nucleotide sequences correspond to the degenerate oligonucleotide probe are underlined.

pendicular. The poly(glycine)-rich region in MSI31 may participate in the formation of β -pleated-sheet structures and the C-terminal acidic domain in the binding of calcium ions or proteins. Two cysteine residues in the N and C termini of MSI31 would also participate in the formation of intermolecular disulphide bonds in the prismatic walls.

In situ hybridization shows that the outer epithelia of the pallial of the mantle expresses MSI60 messenger RNA (Fig. 2a), and that the outer epithelia of the mantle edge expresses MSI31 mRNA (Fig. 2b). The pallial of the oyster mantle also expresses

mRNA for a soluble protein found in the nacreous layer that has carbonic anhydrase activity¹⁰. These findings suggest that the proteins in the pallial construct the nacreous layer and the proteins from the edge of the mantle construct the prismatic layer. As MSI60 and MSI31 have no carbonic anhydrase active-site sequence and no *N*-glycosylation site, these proteins may selectively bind soluble aspartate-rich matrix glycoproteins^{5,6} and carbonic anhydrase¹⁰ responsible for the formation of CaCO₃ crystals, after they construct the frameworks of the nacreous and prismatic layers⁶.

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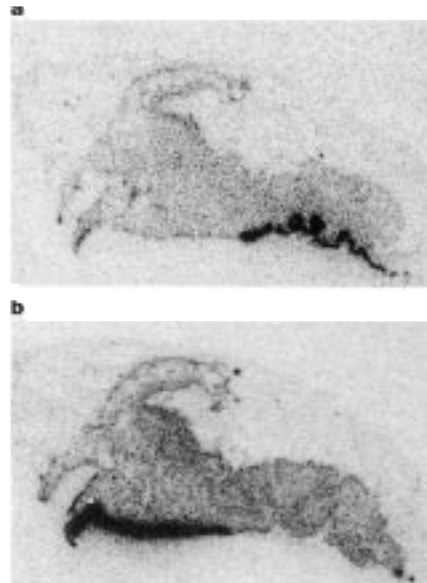


Figure 2 *In situ* hybridization of **a**, MSI60 and **b**, MSI31 mRNA in the mantle of *P. fucata*. The edge of the mantle is to the left and the outer epithelial region is at the bottom.

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Speculation on population

Matters of Life and Death: Perspectives on Public Health, Molecular Biology, Cancer, and the Prospects for the Human Race by John Cairns
Princeton University Press: 1997. Pp. 257.
 \$29.95, £23

 Joel E. Cohen

During his distinguished scientific career, John Cairns served as director of Cold Spring Harbor Laboratory on Long Island, New York, director of a laboratory of the Imperial Cancer Research Fund in London, and professor at Harvard School of Public Health in Cambridge, Massachusetts. He conducted and led research in molecular biology, cancer and public health. These three fields are the subject of two chapters each in this collection of six essays. The essays aim "to give a simple description of some of the great developments in the biological sciences, including the science that underlies the preservation of human health... the essays are designed to be read by people who know nothing about science".

Cairns achieves these goals. His lucid exposition shows how experiments, observations and calculations support some of the grand conclusions of modern biology. He offers fresh — although sometimes controversial — insights. I found the book an enjoyable way to learn more about my own and other fields.

Cairns's views on molecular biology and cancer are well known because of the wide attention given to his papers and his book *Cancer: Science and Society* (W. H. Freeman, 1978). At the editor's request, I therefore limit my comments to his views on mortality, population and the future of the human species, as presented in his first and last chapters.

Cairns's first chapter is a history of mortality. Worldwide, from the time of Palaeolithic hunter-gatherers to the middle of the eighteenth century, the average human life hardly exceeded 40 years anywhere. In seventeenth-century Breslau in Poland, as in Rome during the empire, most newborns did not survive long enough to have a child, and life expectancy was about 26 years.

In the eighteenth century, death rates began to fall, at first slowly and only among aristocracies. In subsequent centuries, the fall in death rate spread around the world and accelerated notably after the Second World War. Global life expectancy rose to 64 years by 1990–95, and as high as 80 years in Japan.

Why did the fall in death rates begin in the eighteenth century? Cairns mentions various theories: an increased consciousness of death rates stimulated by then-new statistical data and methods; an increased concern by governments for the welfare of the population as

a basis for taxation and military strength; better nutrition; a declining virulence of infectious diseases; and a host of other improvements under human control such as vaccination, medical hygiene, increased trade, greater crop diversity, better buildings that excluded rats with plague, more washable fabrics for clothes, and declining fatalism about the lot of humans. From the very beginning and still today, within and among nations, the poor suffered higher death rates than the rich.

Cairns offers provocative speculations about the future of mortality. "For the rich nations it seems unlikely that there can be much more change in the pattern of mortality, because they have already attained a life expectancy that takes most of the population to the brink of senility. What these countries now have to do is learn how to run a nation where 25 per cent of the population are receiving pensions and expensive health care and are too old to work, 20 per cent are too young to work, and there is less and less work for most of the remaining 55 per cent." This forecast of little change reminds me of the remark in 1972 by Sir Macfarlane Burnet and David White that "the most likely forecast about the future of infectious disease is that it will be very dull".

Mortality change in today's poor countries is unlikely to retrace the path of mortality in the now-rich countries, Cairns argues, because poor countries are experiencing an unprecedented speed and magnitude of demographic change, because the tropical countries have a greater abundance of potential vectors of infectious diseases, and because the poor countries have to suffer advice and interventions from the rich.

Cairns illustrates this last problem with a startling graph taken from a 1983 paper by J. T. Hart. For 19 industrialized countries, the figure shows the number of doctors per 10,000 population on the abscissa and, on the ordinate, the residual of infant mortality after adjusting for a simple linear relationship with gross national product (GNP) per person. "You might guess, for example, that the more doctors you have, the lower will be infant mortality" after adjusting for GNP per person. The data demonstrate the opposite.

Japan and Finland in the lower left corner have the smallest number of doctors per person and the lowest adjusted infant mortality; Italy, West Germany and Austria in the upper right corner are highest on both variables. "This does not, of course, mean that doctors are a cause of death in babies. Rather it suggests, to me at least, that countries can either



Bleak houses

"Beneath Scoresbysund's benign appearance from the air is a wild native village with high rates of violent crime, alcoholism, and suicide." This view of a tarnished wilderness in Greenland is one of many striking

photographs in *Poles Apart: Parallel Visions of the Arctic and Antarctic* by Galen Rowell. The book is now available in paperback from University of California Press (\$24.95, £20).

manage infant and maternal care at the national level (which is mainly a matter of prenatal care and does not require advanced technology) or they can choose to wash their hands of the whole thing and let market forces determine what happens. And market forces will lead to an overabundance of doctors... we should advise developing countries to go for the inexpensive forms of public health... rather than [imitate] Italy and West Germany."

This interpretation, while plausible, is not conclusive. Would infant mortality (adjusted for GNP per person) decline, as Cairns predicts, if public health expenditures or the fraction of infants who received prenatal care were plotted on the abscissa instead of doctors per person? If infant mortality were adjusted for other variables in addition to GNP per person, would the relationship with the number of doctors per person change? It seems difficult to argue with Cairns's general conclusion: "The most important message of this chapter is simply that whenever statistics are available, it is folly not to use them." But I would add the proviso, "carefully".

Cairns's final chapter, "Population", has three parts. The first reports how Malthus's views on the relative importance of the positive and preventive checks on population evolved as he collected relevant data. The second part is a brief history of "the explosion in the populations of *Homo sapiens*". In England, up to the beginning of the nineteenth century (just when Malthus was writing), population and prices moved hand in hand. As the number of people increased, the cost of living increased proportionately. So population growth led to scarcity of resources. As population and prices rose, life expectancy fluctuated erratically between 35 and 40 years. So the Malthusian view that rising population would call forth positive checks (rising death rates) in response to scarcity was not true for England up to the time Malthus wrote. Rather, rates of marriage changed, thereby adjusting fertility in response to changes in wages (a preventive check).

Between 1750 and 1850, England, Sweden and France experienced completely different trajectories of birth and death rates. These different responses to different social and economic circumstances, even within the cultural sphere of Western Europe, make the search for universal models of demographic change much more difficult.

The third part of the chapter deals with "Climate and the long-term prospects for the human race". Cairns gives examples to "suggest that all the technology in the world may be hard pressed to protect us from the effects of a really massive change in climate. What Malthus might have called the ultimate danger is that the Earth's climate may, for all we know, be delicately poised and easily pushed in one direction or the other.... The worry, therefore, is that the changes we are causing in

the Earth's atmosphere may unlock the Earth from its present accommodating pattern of climate."

Cairns reproduces a graph of average global temperature (inferred from ice cores) over the past 80,000 years. It shows that the stable warmth experienced over the past 10,000 years is exceptional. Cairns suggests that humans may rock the climatic boat, but another possibility is that the climatic boat may rock for reasons humans simply do not understand.

This final chapter is a curious mix of certainty and doubt, of optimism and gloom. It opens with a flat assertion: "Sooner or later every nation will have gone through the demographic transition and have adjusted to its new, reduced death rate by lowering its birth rate." I wonder how he knows that.

From 1987 to 1995, male life expectancy in Russia fell from 65 to 58 years as male death rates rose. Maurice King has forecast demographic entrapment, in which poor countries so degrade their natural environments that they are unable to lower death rates. Bernard Gilland has warned of the possibility of a demographic interlude, in which death rates fall and then, because birth rates do not fall rapidly enough, death rates rise again.

I think Cairns's forecast is quite likely, but hardly inevitable, because (I hope) both the wealthy and the poor will eventually see that it is in their self-interest to create the conditions that will make the demographic transition happen everywhere. For the world today, the demographic transition is a choice, not an inevitability.

On the very same page, Cairns jumps from the optimistic certainty of his opening sentence to doubtful gloom: "Unfortunately, as a result of the Industrial Revolution, humankind has acquired a heightened ability to consume resources, and this great proliferation.... will bequeath to the Earth as a whole a legacy from which it may never recover... [Unlike] the collapse of the Mayan civilization, this time the disaster promises to be on a global scale." I wonder how he knows that.

In discussing population, Cairns's tone is highly charged: "The way we despoil the world... is the product of two factors... human behavior [and] the number of humans whose behavior has to be modified. Yes, the average inhabitant of the Western world consumes too much; but that is bad for the Earth because there are already too many of these consumers and soon there are going to be many more." On reading these lines, some people will nod agreement, and some will shout denial, but the analytical question of what constitutes "too much" and "too many" has not been answered.

In another unanalysed claim, Cairns states a theory of human fertility: "Like many animals, humans tend to adjust their rate of reproduction to match what they believe will be the opportunities for the next generation."

There is no supporting citation. I wonder how he knows that.

Scientists value conceptual analysis and factual support for their own sake. A broader social value of analysis and facts is that they provide credible common ground for moving towards consensus and action. The Cato Institute (on the 'population right') and Negative Population Growth (on the 'population left') can agree that the 1.2 billion people in the world with 1995 average GNP per person of US\$19,300 have a population doubling time, at 1997 growth rates, of around 560 years, while the other 80 per cent of the world's population (4.5 billion), with 1995 average GNP per person around US\$1,100, would double in 38 years at 1997 growth rates. They can agree that more people eat better now than ever before, but that as many as 2 billion are malnourished and 0.8 billion are undernourished.

They can agree that infant mortality rates globally are lower than ever before, but a newborn in the poor world has a chance of dying before its first birthday about seven times higher than that of a newborn in the rich world. They can agree that more people are literate than ever before, but that approximately a billion are illiterate and two-thirds of those are women.

Neither complacency nor alarm can replace factual analysis of the interactions among populations, economics, the environment and cultures. □

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Nostalgia for the USSR

Essays of a Soviet Scientist: A Revealing Portrait of a Life in Science and Politics by Vitalii I. Gol'danskii

AIP Press: 1997. Pp. 303. \$32.95, £ 27.50

Zhores A. Medvedev

Until recently, the former Soviet Union was perceived as an extremely centralized superpower. But within it there were parallel hierarchical pyramids of rank and profession in which people aspired to rise throughout their lives. The Academy of Sciences of the USSR was the highest and most stable of those pyramids, offering great privilege and honour to those at its apex.

Not only the most famous scientists but also those who were most loyal to the Soviet regime were elected to the ranks of the academy's 300 life members. In the past 50 years only one political dissident — Andrei Sakharov — emerged among the members



Bastion of privilege: the USSR's academy of sciences showered rewards on scientists loyal to the regime.

of the academy. Members were given the title 'academician' and usually became directors of scientific institutes irrespective of whether or not they were proficient organizers. They also received a second salary from the academy, moved into academy apartment blocks in Moscow or to special 'academy' towns, were provided with large state-owned 'academy' dachas, had the permanent use of 'academy' cars and chauffeurs, received medical treatment in élite 'academy' hospitals and took holidays in 'academy' sanatoria and resorts.

The Academy of Sciences was considered so important that the former president Mikhail Gorbachev gave it the right to elect 30 of its members to the new Soviet parliament, the Congress of People's Deputies, when he reformed the electoral system in his programme of democratization. Academician Vitalii Gol'danskii became one of those deputies in 1989.

One of the privileges enjoyed by an academician was the right not only to publish his papers without peer review, but also to have the academy republish them as separate volumes of collected works towards the end of his life. At some 'significant' date after his death, the academy would also publish a book of recollections written by pupils, colleagues and friends. Pupils usually discussed his scientific endeavours at the institute, for which the great scientist had received a Stalin or Lenin prize, or even the title 'Hero of Socialist Labour'. Friends wrote about the things he did outside the institute.

The first third of *Essays of a Soviet Scientist* consists of extracts from Gol'danskii's contributions to such books — an appreciation of his teacher, Academician Nikolai Semenov, and reminiscences of his friends, Academicians Lev Artsimovich, Lev Landau, Yakov Zel'dovich and others. The rest is made up of short sketches, essays, articles and interviews reprinted from newspapers and journals published between 1968 and 1994. Uncon-

nected by any central idea, they cover a variety of themes, ranging from music and poetry to the problems of nuclear disarmament.

But none of them attempts to elucidate the most acute problem about which Gol'danskii must have known a great deal more than all his readers — why it is that the Academy of Sciences of the USSR, the richest and most prestigious scientific centre in the world, previously the leading research centre in many aspects of atomic physics, mathematics, chemistry, astrophysics and the study of the cosmos, has been saved from complete collapse in the past five years only by grants from George Soros, and by renting out the buildings of its scientific institutes to businesses and foreign companies. □

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Squabbles in space

A New Force at a New Frontier: Europe's Development in the Space Field in the Light of its Main Actors, Policies, Law and Activities from its Beginnings up to the Present
by Kevin Madders

Cambridge University Press: 1997. Pp. 604.
£95, \$150

Roy Gibson

Many of us who were involved with the European Space Research Organization (ESRO), the European Launcher Development Organization and the European Space Agency (ESA) in the 1960s and 1970s will regret not then having had the advantage of reading Kevin Madders's book. It explains many things that were not clear, at least to me, during the early hectic years.

So far as I can judge, it is accurate and painstakingly complete, particularly in its account of the first two decades of European

space activities. But one should not expect a compulsive, cover-to-cover read; it is much more a reference book to be consulted by those who have an interest in the legal, political or social aspects of European space.

Because the author has decided not to have appendices, the narrative (or narratives, because there are several interwoven themes which Madders does well to keep apart) is delayed by the inclusion in the main text of long extracts from contemporaneous documents. All are apposite and authentic, but it makes hard going unless the reader happens on an aspect in which he or she has a special interest or knowledge. The conclusions at the end of each chapter could be read serially, but that would not make a really satisfactory overview.

One upshot of the chosen presentation is that one has to read more than 200 pages before receiving the assurance that ESRO did in fact, amid the political haggling and mutual blackmailing, manage to develop some satellites and to have them launched into orbit. But a discouragingly large proportion of time was indeed spent in committee meetings of representatives of the member states at various levels. One of the main problems of that time was to insulate the engineers and scientists from political interference, and to allow them to get on with their work.

The author points to the continued absence of a truly European space programme or strategy, in spite of the provision in the ESA convention that intended the agency to be the means of coordinating European and national space programmes into something approaching a coherent whole. That goal may have been possible in the scientific sector. But, once the industrial and commercial value of application satellites, such as communication satellites, became apparent, the larger member states ignored that part of the convention. They concentrated instead on pushing and pulling the direction and content of the ESA programme so that it furthered national ambitions, while leaving them free, individually or in combination, to develop their own satellites in areas where the commercial promise was the greatest. That remains true today.

For my taste, the analytical approach taken by Madders has obscured the essential change in the nature of space activities, not only in Europe, over the past ten or so years. Government support for scientific satellites remains, although they need to compete for funding with other scientific areas. Governments are weary of investing in space spectacles, either in-orbit infrastructure such as the International Space Station or planetary exploration programmes to Mars and the like. The return, either in the form of direct benefits or through bursts of public enthusiasm, is too

uncertain in an age in which public funding overall is in short supply.

The emphasis is therefore increasingly on what Madders calls "useful space". This, by definition, means space activities that impinge on other sectors of life ranging from telecommunications and television transmission protection, through navigation and location services to environmental protection, land use, treaty and convention verification, and so on. The end-users are not in the space community, and indeed the space element is often only one of many components. Not surprisingly, science ministries are not keen to shoulder the costs indefinitely. It follows, therefore, that future projects need to be specified and at least partially funded outside the space agency circuit. Even in meteorology, where the use of space techniques is seldom contested, it has been difficult to persuade the end-users to take over funding responsibility from the science ministries; it is considerably harder in other fields.

The space agencies still have an important contribution to make even in the area of "useful space", but it is one that has to be carefully negotiated with the new users and funders. Aerosat was a worthy international project in the 1970s, but it failed mainly because the end-users were not behind it.

This change of emphasis is reflected in the functions and status of space agencies, and in many parts of the world they have lost a lot of their former influence. Although this point is left until very late in Madders's book, it is brought out by a description of the increasing role of the European Commission in the development of European space science. The very balanced treatment shows that this is not simply a story of rivalry between the commission and ESA, and that the newcomer can in fact be helpful to the future development of space science, particularly in the complicated area of "useful space" where public and private sectors have to learn to operate together more efficiently.

Madders admirably controls his philosophical yearnings until the last few pages, and only then does he allow himself a few brave words about the Space Age's most valuable gift being the "power it gives us to shape a future of greater choice".

In moments mellowed by a judicious liquid intake, I can agree with him. But for the rest of the time (and this is still the larger portion) I am oppressed by the continuing petty squabbles among European politicians in our crab-like movement towards a stronger Europe, and their inability to spend enough time on a more impartial assessment of the advantages of meaningful cooperation in European space. I only hope that Madders's optimism is justified. □

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Creatures of mystery

Snakes in Question: The Smithsonian Answer Book by Carl H. Ernst and George R. Zug
Smithsonian Institution Press: 1997. Pp. 203. \$49, £38.25

Kaleidoscopic Tree Boas: The Genus Corallus of Tropical America by Peter J. Stafford and Robert W. Henderson
Krieger: 1996. Pp. 86. \$28.50, £26.50

Coral Snakes of the Americas: Biology, Identification, and Venoms by Janis A. Roze
Krieger: 1996. Pp. 328. \$95, £86.95

Venomous Snakes: Ecology, Evolution and Snakebites by R. S. Thorpe, W. Wüster and Anita Malhota
Oxford University Press: 1996. Pp. 276. £75, \$145

Snakes: The Evolution of Mystery in Nature by Harry W. Greene
University of California Press: 1997. Pp. 351. \$45, £35

J. L. Cloudsley-Thompson

Contrary to popular belief, the majority of snakes, including many of the most venomous, tend to be timid and retiring. Like Greta Garbo, they prefer to be left alone. By contrast, human beings find it difficult to leave snakes to their own devices. They arouse scientific curiosity and our aesthetic sense because many of them are so beautiful. In some people as well as certain wild animals, they engender a sense of fear. All these interests are catered for in these books.

In a chapter on folk tales, Carl Ernst and George Zug point out that no animal is the subject of so many myths and half-truths as is the snake, to which human beings attribute symbolic and erotic meanings. *Snakes in Question* sets out to answer the questions most frequently asked about snakes by both adults and children. It also offers insight into their basic biology and contains useful data such as the body sizes of selected species and their speed of locomotion. It may reassure some people to learn that no snake can move half as fast as a human being.

Kaleidoscopic Tree Boas covers the natural history and captive management of the genus *Corallus*, a small group of highly-adapted tree-dwelling snakes whose striking appearance and often exorbitant coloration are clearly illustrated in 53 plates. Keys to the four known species and diagnoses of their subspecies are provided in this useful little guide.

Far more ambitious and technical is the monographic treatment of the *Coral Snakes of the Americas* by Janis Roze. Not only are full descriptions of nearly 150 species and subspecies given, but maps of their distribu-

tion and variations are provided. Also included are chapters on morphology, ecology, feeding, food, cannibalism, reproduction, enemies and defence. There is a section on venoms and their effects, an extensive bibliography and detailed indexes.

The chapter on mimicry is especially interesting as it reviews the long-standing problems presented by false coral snakes and the 'deadly model' paradox. Mimicry relationships represent a wide array from strict batesian to extreme mullerian mimicry. Following Harry Greene and R. McDiarmid, the so-called 'mertensian' is regarded as a version of mullerian mimicry and not as a different category of mimicry.

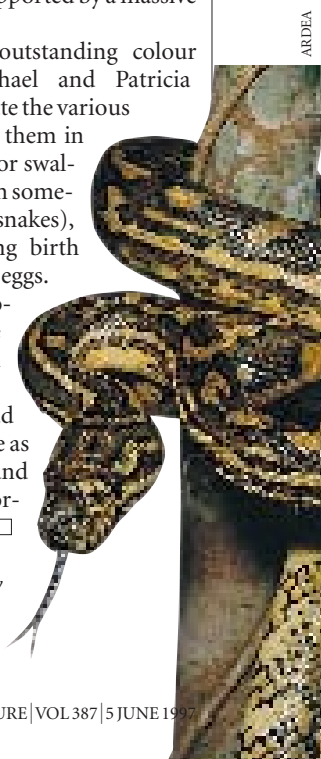
Venomous Snakes is the latest volume of Symposia of the Zoological Society of London, and maintains the customary high standard of that series. The intention of the symposium's organizers was to bring together leading researchers on all aspects of venomous snake biology to discuss a wide range of topics and forge stronger links among themselves. The 18 chapters are authoritative, specialized and diverse. Their subjects range from the systematics of sea snakes and the DNA evolution of pit vipers of the genus *Bothrops* to a review of phospholipases in snake venom. The impact of molecular methods for the reconstruction of phylogenetics is apparent in six of the nine papers devoted to mainly taxonomic subjects.

For most herpetologists — and zoologists in general — the most interesting of these books will be Harry Greene's *Snakes: The Evolution of Mystery in Nature*. This is by no means a superficial account, as its title might suggest, but an extremely well-written and scientific introduction to the evolutionary biology of snakes, interwoven with folklore and original observations on their natural history, supported by a massive bibliography and index.

The hundreds of outstanding colour photographs by Michael and Patricia Fogden not only illustrate the various species but often show them in action — envenoming or swallowing their prey (which sometimes includes other snakes), fighting, mating, giving birth and hatching from eggs. Most of these photographs were taken in the wild, in 18 countries on six continents.

This is a book to read for interest and pleasure as well as for accurate and original scientific information. □

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ARDEA

Functional rafts in cell membranes

Kai Simons & Elina Ikonen

A new aspect of cell membrane structure is presented, based on the dynamic clustering of sphingolipids and cholesterol to form rafts that move within the fluid bilayer. It is proposed that these rafts function as platforms for the attachment of proteins when membranes are moved around inside the cell and during signal transduction.

In the prevailing view of cellular membrane structure, lipids in the bilayer function mainly as a solvent for membrane proteins¹. But in the fluid bilayer, different lipid species are asymmetrically distributed over the exoplasmic and cytoplasmic leaflets of the membrane². The lipids are also organized in the lateral dimension and impose more short- and long-range order than was previously recognized³. This lateral organization probably results from preferential packing of sphingolipids and cholesterol into moving platforms, or rafts, onto which specific proteins attach within the bilayer. We now propose a model for membrane structure that describes the organization of these lipid microdomains and we present evidence that proteins can selectively be included or excluded from these microdomains. We conclude that the function of these microdomains is to serve as rafts for the transport of selected membranes or as relay stations in intracellular signalling.

The concept

In epithelial cells, plasma membranes are polarized into apical and basolateral domains⁴, with the former being enriched in sphingolipids (glycosphingolipids and sphingomyelin) and the latter in the glycerolipid phosphatidylcholine^{2,5}. Mixing of sphingolipids and phosphatidylcholine, both of which are localized to the exoplasmic leaflet, is prevented by the tight junctions between the two cell-surface domains^{2,5}.

Studies on the delivery of newly synthesized sphingolipids in epithelial MDCK cells showed that a simple glycosphingolipid, glucosylceramide, was preferentially transported to the apical membrane^{5,6}. To explain delivery to the apical domain, it was proposed that glycosphingolipid clusters formed within the exoplasmic leaflet of the Golgi membrane⁵: these microdomains were considered to be sorting centres for proteins destined for delivery to the apical plasma membrane. In support of this model, glycosylphosphatidylinositol (GPI)-anchored proteins use glycolipid anchors as apical sorting determinants⁷. Moreover, caveolae, small invaginations of the plasma membrane that are devoid of clathrin coats and are present in many cell types⁸, contain clusters of glycosphingolipids⁹ and need cholesterol to function¹⁰. As caveolae may be involved in endocytosis⁹ and transcytosis¹¹, a function for sphingolipid rafts in membrane trafficking was plausible¹².

General properties of raft lipids

A model for the organization of sphingolipid-cholesterol rafts is shown in Fig. 1 (and see Table 1). In this model, sphingolipids associate laterally with one another, probably through weak interactions between the carbohydrate heads of the glycosphingolipids. The sphingolipid head groups occupy larger excluded areas in the plane of the exoplasmic leaflet than do their predominantly saturated lipid hydrocarbon chains. Any voids between associating sphingolipids are filled by cholesterol molecules which function as spacers (Fig. 1b). The close-packed sphingolipid-cholesterol clusters behave as assemblies within the exoplasmic leaflet, where the intervening fluid regions are occupied by unsaturated phosphatidylcholine molecules (Fig. 1a). Glycosphingolipids usually carry a long fatty acid which is amide-bonded to the sphingosine base and can interdigitate with the cytoplasmic leaflet of the bilayer¹³ (but see ref. 14). As cholesterol is present in both leaflets², it functions as a spacer

in the cytoplasmic leaflet as well, filling voids created by interdigitating fatty acid chains (Fig. 1b). The nature of the phospholipids occupying the cytoplasmic side of the microdomain is unknown, but they will probably also be carrying mainly saturated fatty acid chains to optimize packing. Individual lipids may move in and out of the rafts³, explaining why sphingolipid-cholesterol clustering is difficult to detect spectroscopically¹³.

Detergent-insoluble complexes

Sphingolipid-cholesterol rafts are insoluble in the detergent Triton X-100 at 4 °C, in which they form glycolipid-enriched complexes^{7,15}. Because of their high lipid content, these detergent-insoluble, glycolipid-enriched complexes (DIGs) float to a low density during gradient centrifugation⁷, which enables any associated proteins to be identified and distinguishes DIGs from other detergent-insoluble complexes. Milder detergents such as octylglucoside will solubilize lipid rafts¹⁶. Glycosphingolipids are insoluble by themselves, and sphingomyelin is resistant to detergent extraction in the presence of cholesterol¹⁶. One problem with Triton X-100 extraction is that the original subcellular locations of DIGs are unknown. Confusion has been created because sphingolipid rafts in apical transport vesicles, in caveolae, and even in plasma membranes from cells devoid of morphologically recognizable caveolae, all form DIGs after Triton X-100 extraction^{17–19}. Only by isolating specific organelles before detergent extraction can the origin of DIGs be determined; neither can it be assumed that two proteins are in contact with each other on the basis of their partitioning into DIGs.

Proteins associated with rafts

Lipid rafts can bind proteins (Fig. 1a): GPI-anchored proteins, transmembrane proteins^{17,18,20} and doubly acylated tyrosine kinases of the Src family²¹ all associate with rafts and are incorporated into DIGs. For example, influenza virus haemagglutinin, an apical

Table 1 Biophysical studies of sphingolipid and cholesterol interactions

Technique	Observation	Ref.
Freeze-etch immunoelectron microscopy	Clustering of asialo-GM1 and Forssman glycolipids	59
Fluorescence microscopy	Co-capping of GM3 and GM1 gangliosides	60
Differential scanning calorimetry	Higher transition temperatures for sphingolipids from the gel to liquid crystalline phase than for glycerolipids	61
Surface pressure-molecular area isotherm studies of lipid monolayers	Increased packing density of sphingolipids and sphingolipid-cholesterol mixtures as compared to mixtures of glycerolipids	62
Fluorescence spectroscopy of non-perturbing fluorescence lipid probes	Sphingolipids form domains in liquid crystalline bilayers of unsaturated phosphatidylcholine	63
Spin-label electron resonance spectroscopy	Cholesterol associates more readily with sphingomyelin than with glycerolipids	64
Nuclear magnetic resonance spectroscopy	Self-association of cerebroside	65; but see ref. 13

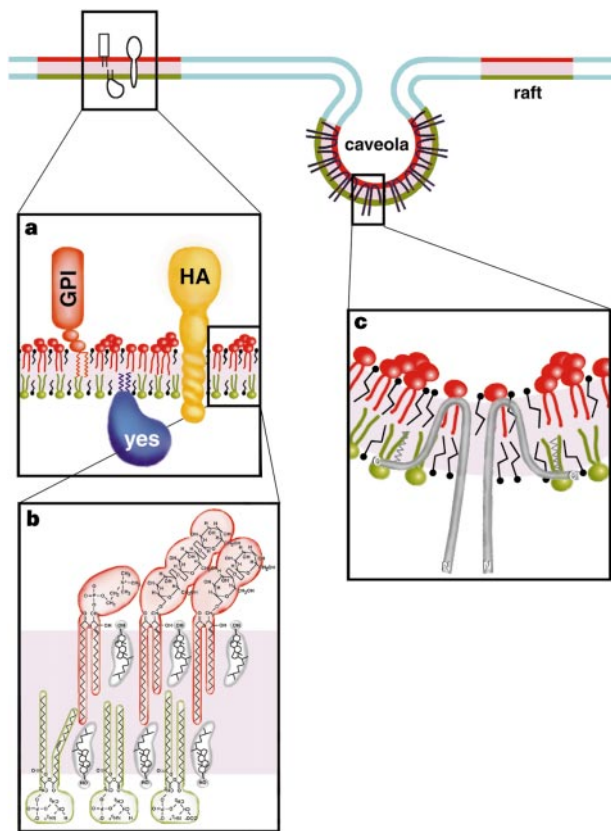


Figure 1 Model for the organization of rafts and caveolae in the plasma membrane. The rafts (red) segregate from the other regions (blue) of the bilayer in which unsaturated phosphatidylcholine is predominantly in the exoplasmic leaflet, which has a different organization of intercalated cholesterol from that in the rafts⁶⁶. **a**, Rafts contain proteins attached to the exoplasmic leaflet of the bilayer by their GPI anchors, proteins binding to the cytoplasmic leaflet by acyl tails (the Src-family kinase Yes is shown), or proteins associating through their transmembrane domains, like the influenza virus proteins neuraminidase and haemagglutinin (HA) (these proteins associate with DIGs in virus membranes). However, if cholesterol is removed by cyclodextrin extraction from the envelope, the virus glycoproteins become soluble in Triton X-100 at 4 °C (P. Scheiffele and K.S., unpublished results). **b**, The lipid bilayer in rafts is asymmetric, with sphingomyelin (red) and glycosphingolipids (red) enriched in the exoplasmic leaflet and glycerolipids (for example, phosphatidylserine and phosphatidylethanolamine; green) in the cytoplasmic leaflet. Cholesterol (grey) is present in both leaflets and fills the space under the head groups of sphingolipids or extends the interdigitating fatty acyl chain in the apposing leaflet. **c**, Caveolae are formed by self-associating caveolin molecules making a hairpin loop in the membrane. Interactions with raft lipids may be mediated by binding to cholesterol and by acylation of C-terminal cysteines⁹.

transmembrane protein¹⁷, and GPI-anchored proteins⁷ become incorporated into DIGs after being transported from the endoplasmic reticulum (ER) to the Golgi complex and then remain insoluble. This delay in DIG association during intracellular transport may be due to the absence of sphingolipid–cholesterol rafts in the ER. Although cholesterol is synthesized in the ER, raft assembly can occur first in the Golgi complex, where sphingolipids are synthesized². Investigation of the association of GPI-anchored proteins with rafts has shown that extraction of cholesterol by saponin renders these proteins soluble in Triton X-100 at 4 °C (ref. 22). Also, interaction with raft lipids confers detergent insolubility on a GPI-anchored protein¹⁶, and the association of GPI-anchored proteins with DIGs *in vivo* is affected by cholesterol and sphingolipids^{23,24}.

For GPI-anchored proteins, the lipid anchor with its two (usually) saturated fatty acyl chains determines raft association²¹. Double acylation by saturated chains, as in Src-family kinases, may determine preferential partitioning into rafts. For transmembrane proteins, membrane-spanning domains can confer an affinity for rafts²⁵. Another membrane protein found in DIGs is caveolin⁸: this localizes to caveolae and to post-Golgi transport vesicles and binds cholesterol, which may explain its association with DIGs²⁶ (Fig. 1c).

Role of rafts in membrane trafficking

Biosynthetic traffic. The intracellular transport of sphingolipid–cholesterol rafts has been studied in the MDCK cell line. It is the apical route that preferentially transports sphingolipid–cholesterol rafts carrying GPI-anchored proteins and apical transmembrane proteins (Fig. 2). Like GPI anchors²⁷ and specific membrane-spanning regions²⁵, *N*-glycans may also be used as apical sorting signals²⁸. Some proteins may depend solely on binding to their *N*-glycans to raft-associated lectins²⁹ for apical delivery, whereas others with membrane anchors could partition into the rafts directly.

But basolateral delivery depends on signals originating from the cytoplasmic domains of basolaterally targeted proteins. These signals usually involve tyrosine or dileucine motifs, as do clathrin-coated-pit signals³⁰. As basolateral proteins are also *N*-glycosylated,

how can this be reconciled with *N*-glycans being apical sorting determinants? It is likely that most cytoplasmic basolateral signals have a higher affinity for their adaptors than do *N*-glycans for the putative apical lectins. Basolateral proteins lacking their cytoplasmic tails are sorted to the apical side, presumably as a result of their carbohydrate chains linking to apical lectins^{28,30}.

As well as having different sorting signals, apical and basolateral delivery in MDCK cells differs at the stages of vesicular docking and fusion³¹: the basolateral route uses the same type of machinery as that described for ER–Golgi, intra-Golgi and synaptic vesicle transport³², whereas apical delivery uses a different docking and fusion mechanism that is still poorly defined³¹ (Fig. 2).

Fibroblasts that have no cell-surface polarity also have apical and basolateral cognate routes from the *trans*-Golgi network (TGN) to the plasma membrane^{33,34} (Fig. 2). The same sorting signals that in MDCK cells direct proteins to basolateral transport vesicles also act in transport from the Golgi to the cell surface. Moreover, there are different vesicular docking and fusion machineries in fibroblasts as well as MDCK cells³⁴.

Endocytic traffic. Sphingolipid–cholesterol rafts are involved in transporting proteins in the endocytic pathways. Endocytosis from the apical surface involves both the clathrin-coated vesicle pathway and another less well defined route to endosomes underlying this membrane domain³⁵. There are no caveolae on the apical membrane of enterocytes³⁵ or of MDCK cells (R. Parton, personal communication). Therefore, caveolae cannot be responsible for the clathrin-independent route. Instead, shallower surface invaginations (72–250 nm deep) may endocytose GPI-anchored proteins³⁵.

Caveolae are found, together with clathrin-coated pits, on the basolateral surface of MDCK cells (R. Parton, personal communication). In contrast to coated pits, which are constitutively endocytosed, caveolae remain attached to the plasma membrane in most cell types, with their release being effected by unknown signals. In endothelia, caveolae are involved in transcytosis across the cell layer^{11,36}. Potocytosis, another pathway of internalization³⁷, is regulated by the reversible closing and opening of caveolae and

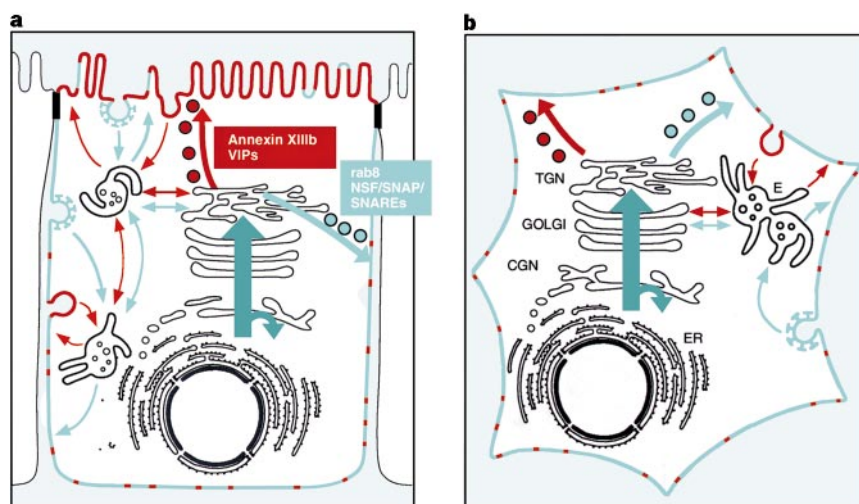


Figure 2 Two postulated post-Golgi circuits in MDCK cells (**a**) and fibroblasts (**b**). Sorting in the raft circuit (in red) is based on sphingolipid-cholesterol microdomains functioning as sorting platforms to which vesicular integral membrane proteins (VIPs) associate as stabilizers or as linkers to other proteins in the raft²⁹. The other circuit (in blue) employs sorting signals in the cytoplasmic tails as well as adaptor and coat proteins binding to them. The NSF-SNAP-SNARE-Rab system is used for vesicular docking and fusion in this blue circuit; in the red circuit specific annexins may be involved⁶⁷. VIPs are also in the basolateral vesicles in MDCK cells but their physical state is different from that in apical vesicles, where they are part of a large protein-lipid complex (K.S. and H. Virta, unpublished results). The red regions of the plasma membrane are sphingolipid-cholesterol rafts and the blue areas are PC-enriched regions. The exact arrangement of the post-Golgi traffic map, including the return routes to the TGN, is still under investigation.

can also internalize SV40 virus and deliver it to the ER³⁸. Both caveolar endocytosis and transcytosis are blocked by agents that sequester cholesterol^{10,36}, although clathrin-mediated endocytosis is unaffected³⁹.

Two post-Golgi circuits?

We suggest that there are two routes for membrane traffic which connect the Golgi complex with the cell surface. One transports membrane proteins that have sorting signals in their cytoplasmic domains³², the other handles sphingolipid-cholesterol rafts and proteins associating with them. Proteins will also be endocytosed by raft routes and by clathrin-coated vesicles, and some proteins will cycle back to the Golgi complex. According to the model, the two circuits will intersect in the different organelles involved in post-Golgi traffic (Fig. 2). Merging of the circuits will demand the appropriate sorting and delivery at each site to ensure that raft components are always segregated from proteins tagged by cytoplasmic signals. There are examples of proteins with cytoplasmic signals being reversibly converted into proteins that move into the raft circuit *in vivo*⁴⁰. A raft-associating protein could be shifted to the other circuit by reversible binding to a protein with cytoplasmic sorting signals.

We need to understand how the post-Golgi circuits are adapted to serve different cell types. For instance, the apical and basolateral route maps are not identical in all epithelial cells⁴⁰. In hepatocytes there is no apical route from the TGN to the apical surface. Instead, apical proteins are first transported to the basolateral surface and then delivered by transcytosis to the apical side⁴⁰. This change in the epithelial traffic map could be accomplished by diverting the biosynthetic raft route, connecting the TGN directly with the apical side in MDCK cells or to the basolateral side in hepatocytes by locating the appropriate docking machinery there. Other polarized cells such as hippocampal neurons have a route equivalent to the apical pathway for delivery of proteins and lipids from the Golgi complex to axons, whereas a basolateral cognate route supplies the dendrites⁴¹. An unusual example of a modulated raft circuit is used in the massive delivery of galactosylceramide from the Golgi complex to produce the extensive myelin sheets in oligodendrocytes and Schwann cells which are required for myelination⁴². An interesting instance of preferential handling of proteins with basolateral sorting signals in fibroblasts is the routing of such proteins to the ruffling edge and into growing processes⁴³.

Rafts in membrane signalling

One advantage of the lateral organization of cell membranes is that proteins involved in signal transduction can be confined to different lipid microdomains. Several signalling molecules partition into DIGs^{15,44,45}; clustering of GPI-anchored proteins for example can activate different signalling pathways depending on the cell type.

This clustering could lead to signal transduction in two ways. (1) Enzymes activated in the rafts involved in anchor release might yield water-soluble phospho-oligosaccharides; these would have to be flipped across the bilayer to be active as second messengers in the cytosol⁴⁶. (2) Clustering of GPI anchors might lead to interaction with signal-transducing proteins that then become activated. A potential link is that two Src family kinases, Lck and Fyn, both implicated in T-cell activation, can be immunoprecipitated with GPI-anchored proteins after Triton X-100 extraction at 4 °C (ref. 21). But it is not clear how GPI-anchored proteins in the exoplasmic leaflet of the raft could communicate with doubly acylated tyrosine kinases in the cytoplasmic leaflet.

Trimeric G proteins⁴⁷ and Ras also associate with DIGs. Ras binds to caveolin in its inactive GDP-bound state⁴⁸, suggesting that caveolae are stores for inactive signalling molecules; on the other hand, in its active GTP-bound form Ras facilitates recruitment of Raf to activated EGF receptors in caveolae⁴⁹, indicating that caveolae function as active signalling depots.

Lipids involved in signal transduction have also been localized to DIGs. These include phosphoinositides such as phosphatidylinositol-(4,5)-bisphosphate⁵⁰ and sphingomyelin, but the membrane topology of the parent sphingomyelin is controversial^{51,52}.

In signalling, the roles of caveolae and DIGs are ill-defined¹⁵. Signalling can occur without caveolae, for example in T lymphocytes¹⁹. But caveolae may act as centres from which multiple signalling pathways originate⁴⁴. Clustered GPI-anchored proteins and glycosphingolipids are both enriched in caveolae, suggesting that proteins and lipids associated with sphingolipid-cholesterol rafts might become trapped in caveolae^{15,33}. Our hypothesis is that proteins associating with rafts present in the plasma membrane become fixed in caveolae if they oligomerize. Caveolae would thus be caveolin-induced invaginations of sphingolipid-cholesterol microdomains that trap 'multivalent' proteins or lipid clusters associating with rafts more effectively than 'monovalent' forms. After trapping, the contents of the caveolae could be internalized and removed from the cell surface in a signal-dependent manner⁸.

The general function of raft association in signal transduction may be to concentrate receptors for interaction with ligands and effectors on both sides of the membrane, thus speeding up binding during signalling and preventing inappropriate crosstalk between pathways. Importantly, the association of proteins with rafts can be modulated: some proteins can bind to other proteins in order to be excluded from rafts⁵⁴ and vice versa⁵⁵.

Perspectives

Early observations leading to the proposal that glycosphingolipid microdomains function in apical protein transport in epithelial cells

have been extended to include cholesterol as a co-organizer of the spingolipid domains. Generally, spingolipid-cholesterol rafts may be an essential feature of all organellar membranes involved in biosynthetic and endocytic traffic beyond the ER. Rafts also help ensure specificity and fidelity during signal transduction.

In this view, proteins embedded in the membrane localize either to the clusters of spingolipids and cholesterol or to the intervening fluid regions of glycerolipids. Several proteins are known to depend on cholesterol⁵⁶ or on spingolipids⁵⁷ for activity. Cholesterol influences lipid-protein interactions by increasing the thickness of the lipid bilayer. The increase in cholesterol concentration along the biosynthetic pathway may cause segregation of the proteins, depending on the lengths of their membrane-spanning domains⁵⁸.

It now remains to provide evidence for the functional significance of lipid rafts in membrane trafficking and signalling, assay the postulated segregation of spingolipid-cholesterol clusters from unsaturated phosphatidyl choline molecules in the exoplasmic leaflet of cellular membranes, and to determine the structural basis for optimal packing of proteins and lipids in the lateral and transbilayer organization of microdomains. □

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A new dynamical class of object in the outer Solar System

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Some three dozen objects have now been discovered^{1–5} beyond the orbit of Neptune and classified as members of the Kuiper belt—a remnant population of icy planetesimals that failed to be incorporated into planets. At still greater distances is believed to lie the Oort cloud—a massive population of cometary objects distributed approximately in a sphere of characteristic dimension 50,000 AU (ref. 6). Here we report the discovery of an object, 1996TL₆₆, that appears to be representative of a population of scattered bodies located between the Kuiper belt and the Oort cloud. 1996TL₆₆ has an orbital semimajor axis of 84 AU, and is in an extremely eccentric and highly inclined orbit ($e = 0.58$, $i = 24^\circ$). With a red magnitude ~ 20.9 , it is the brightest trans-neptunian object yet found since Pluto and Charon. Its discovery suggests that the Kuiper belt extends substantially beyond the 30–50 AU region sampled by previous surveys, and may contain much more mass than previously suspected.

The object 1996TL₆₆ was found in the course of a large-area, medium-depth survey of the outer Solar System. Most of the Kuiper-belt objects (KBOs) found to date are near the brightness limit of ground-based telescopes (red magnitude $m_R > 22$), severely restricting opportunities for physical study. One of the goals of the large-area survey is to remedy this situation by searching for larger (brighter) trans-neptunian objects. This search makes use of a unique large-format charge-coupled device (CCD) mounted on the University of Hawaii 2.2-m telescope. The 8K CCD is an array of eight CCDs, each $2,048 \times 4,096$ pixels. The detector was used in 3×3 binning mode, resulting in a scale 0.41 arcsec per binned pixel and a total field of view of 18.4×18.4 arcmin, or 0.09 square degrees. Moving objects are detected in near-real time using a computer algorithm developed specifically for this purpose⁷. Sample images of 1996TL₆₆ are shown in Fig. 1.

On attempting orbital representations from the initial data in October 1996, it was apparent that the orbit of 1996TL₆₆ was quite highly inclined, and it seemed plausible that the object was of the 'Plutino' type². Plutinos resemble Pluto in that they are librators in the 2:3 mean-motion resonance with Neptune (semimajor axis $a \approx 39$ AU), which protects them from close encounters with the planet⁸. The Plutinos make up 30–40% of the known Kuiper-belt population, the other members of which are mostly confined to low-eccentricity, low-inclination orbits with $42 \leq a \leq 46$ AU (refs 8,9). Follow-up observations in December 1996 revealed discrepancies between the recorded positions and the Plutino-type predictions that were too large to be absorbed into a 2:3-resonant orbit solution. It was clear that the object had to be close to the perihelion of a much more eccentric orbit¹⁰. The orbital elements of the best-fit orbit¹¹ are listed in Table 1, along with their estimated uncertainty ranges. Evidently, 1996TL₆₆ exhibits a new type of trans-neptunian dynamics. The distinction between 1996TL₆₆ and known KBOs is

clear in Fig. 2. Previously discovered KBOs have orbits clustered inside ~ 50 AU. In contrast, the large, eccentric orbit of 1996TL₆₆ carries it to more than 130 AU at aphelion, and to ~ 35 AU at perihelion. 1996TL₆₆ provides the first direct evidence that the trans-neptunian population extends much further than the 30–50 AU region sampled by our previous surveys.

1996TL₆₆ was found after examining only 25 square degrees. The discovery of this object in such a small fraction of the sky suggests that, unless we are improbably lucky, it is merely the first detected of a larger population of similar bodies. If objects in the TL₆₆-class are members of a flat belt with inclinations i up to 30° (as was found for the Kuiper belt inside 50 AU; ref. 5), they would occupy a projected area of $\sim 2 \times 10^4$ square degrees. The discovery statistics then imply that ~ 800 objects of this type would be detected if we extended our 8K CCD survey to the entire ecliptic band. With an assumed red geometric albedo of 4%, and heliocentric and geocentric distances of 35.2 and 34.2 AU, respectively, we estimate that 1996TL₆₆ has a diameter of 490 km. With bulk density $1,000 \text{ kg m}^{-3}$, its mass is $6 \times 10^{19} \text{ kg}$ ($10^{-5} M_{\text{Earth}}$; $1 M_{\text{Earth}} = 1$ Earth mass). The combined mass of 800 such objects is $5 \times 10^{22} \text{ kg}$ ($8 \times 10^{-3} M_{\text{Earth}}$). This mass can be scaled to other adopted albedos, p , by the ratio $(0.04/p)^{3/2}$.

There is good reason to believe that this represents an extreme lower limit to the population of similar bodies, as a result of the effects of observational selection^{2,5}. Ours is a flux-limited survey, which preferentially samples the brightest (that is, the largest and closest) objects, and those with low-inclination orbits. For example,

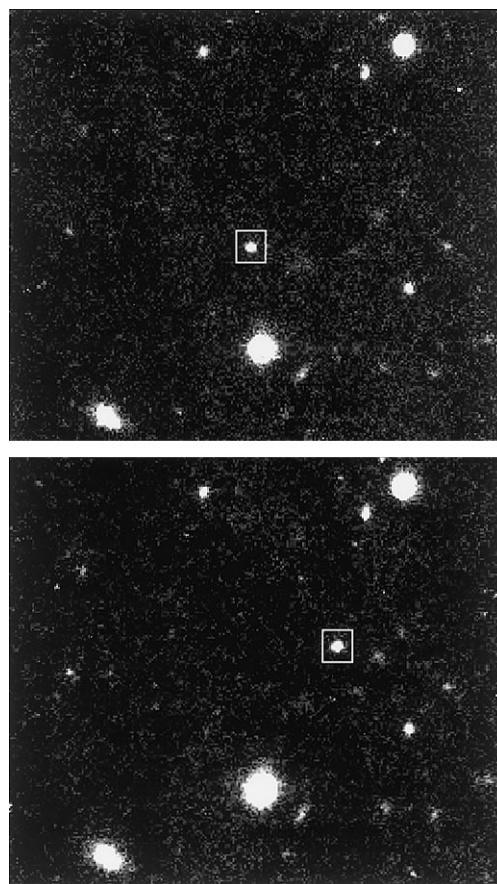


Figure 1 Object 1996TL₆₆ imaged on UT 1996 October 15 at 07:14 (top panel) and 12:41 (bottom panel). The field shown has north to the bottom, east to the left. Note the relative motion westward against the fixed stars. The field of view is 1.6×1.4 arcmin, representing 0.7% of the total area imaged by the 8K CCD mosaic. The integration time was 600 s through a broadband (5,000–7,000 Å) filter.

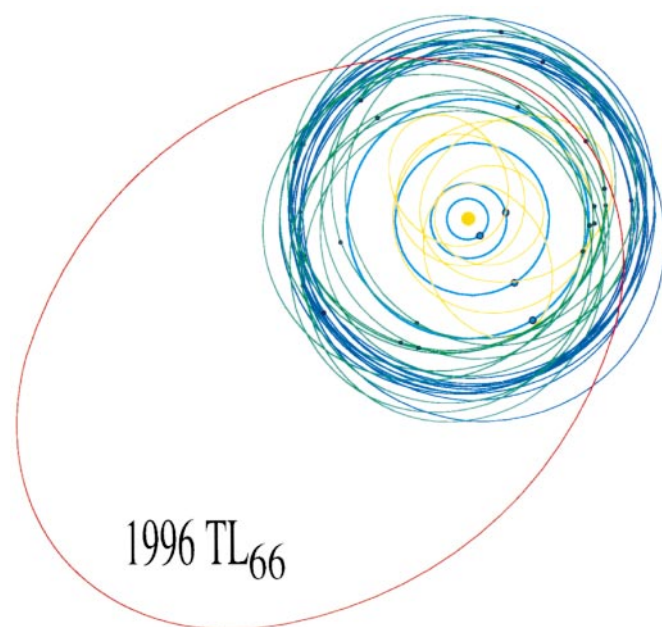


Figure 2 Planar view of the outer Solar System. The four light-blue, nearly concentric orbits are those of the giant planets. The dense band of orbits refers to the best observed Kuiper-belt objects (including Pluto), while the six yellow inner orbits refer to the Centaurs. The large, eccentric orbit of 1996TL₆₆ extends far beyond the previously known Kuiper belt.

1996TL₆₆ itself would only be bright enough to be detected in the present survey during the $\sim 12\%$ of its orbital period centred on perihelion. Very roughly, this suggests that the true population must be ~ 8 times larger than the observable population, that is $\sim 6,400$ objects ($0.07 M_{\text{Earth}}$). Monte Carlo simulations of a set of TL₆₆-like bodies are consistent with this crude estimate.

The total mass of an inverse-cube size distribution is $M = (8/3)\pi\rho N r_{\text{min}}^2 (r_{\text{max}} - r_{\text{min}})$, where $\rho = 1,000 \text{ kg m}^{-3}$ is the bulk density of the objects, and N is the total number of objects in the radius range $r_{\text{min}} \leq r \leq r_{\text{max}}$. If we take r_{min} to be the radius of 1996TL₆₆ and $r_{\text{max}} = 1,100 \text{ km}$ (that is, Pluto-sized), this relation yields a total mass of $2.8 \times 10^{24} \text{ kg}$ ($0.5 M_{\text{Earth}}$) contained in TL₆₆-like objects or larger between ~ 40 and 200 AU . The mass estimated here is larger than the $(0.06\text{--}0.25) M_{\text{Earth}}$ estimated mass of the inner Kuiper belt⁵, but nevertheless consistent with the dynamical upper limit¹⁰ of $\sim 1 M_{\text{Earth}}$ inside 100 AU . The inverse-cube size distribution further predicts $\sim 4 \times 10^8$ objects (comets) with radius $1 \text{ km} \leq r \leq 245 \text{ km}$, in the same volume. These estimates are obviously crude, given the discovery statistics of 1 and the uncertainties in the projected area occupied by the TL₆₆ population; however, there is little doubt that the region out to at least a few hundred AUs is substantially populated.

What is the likely origin of this new class of objects? In his discussion of the Uranus–Neptune region as the likely birthplace of icy conglomerates¹³ in the Oort cloud⁶, Kuiper¹⁴ envisaged that the outer planets gravitationally ejected protocomets from that region onto large eccentric trajectories. This basic idea has been refined by others^{15–17}. These works suggest that the trans-neptunian population should contain two groups of planetesimals: those on primordial near-circular orbits (which we identify with the classical Kuiper belt, albeit modified by resonances in the case of the Plutinos) and those on scattered eccentric orbits (which, for convenience, we will call scattered KBOs). The orbital characteristics of 1996TL₆₆ suggest that it is a scattered KBO. Objects in the classical Kuiper belt are on stable or weakly chaotic orbits and have managed to survive

Table 1 Orbital elements for 1996TL₆₆

	Nominal value	Uncertainty range
Semimajor axis (AU)	83.77	82.4–85.5
Eccentricity	0.581	0.57–0.59
Inclination (deg)	23.948	23.99–23.93
Longitude of ascending node (deg)	217.764	217.75–217.77
Argument of perihelion (deg)	182.59	174.7–187.4
Mean anomaly (deg)	358.54	360.3–357.6

These values are for epoch 1997 June 1.0, equinox 2000.0.

planetary perturbations over the age of the Solar System. The origin of the scattered population is more ambiguous. One possibility is that the scattered KBOs are Uranus–Neptune region planetesimals scattered by planetary embryos into large, eccentric orbits (as part of the process that formed the Oort cloud)^{15–17}. Presumably, this scattered population would merge smoothly with the Oort cloud at larger distances¹⁷. An alternative scenario is that the scattered KBOs originated in the classical Kuiper belt, but have been scattered outwards by a transient population of large planetesimals¹⁸ or by Neptune. In particular, those KBOs perturbed outward by Neptune could spend most of their lifetime trapped in various resonances, resulting in dynamical lifetimes comparable to the age of the Solar System¹⁹. Further observations of 1996TL₆₆ should help to constrain its origin further; for example, there is the possibility that 1996TL₆₆ actually lies in a resonance (for example, the 2:9 resonance at 82 AU)²⁰.

We note that some KBOs lost in previous surveys⁸ may have been of the 1996TL₆₆ type. Objects that clearly have orbits of high inclination, but often completely indeterminate eccentricity, would be the best of such candidates. These might include 1994JV, 1994TH, 1995GJ and 1996KY₁, the available observations of which can easily be satisfied by TL₆₆-like orbits with $i \approx 12, 17, 17$ and 22 degrees, respectively. Such orbits might explain the failure to recover these objects a few months after their discoveries, although it is also possible that some non-recoveries were artefacts of poor seeing, poor transparency, and confusion with stars in the recovery attempts.

1996TL₆₆ provides the first direct evidence of a scattered planetesimal population between the currently known Kuiper belt ($30\text{--}50 \text{ AU}$) and the Oort cloud ($\sim 10^4 \text{ AU}$). Although the origin of these scattered KBOs is still unknown, it is likely that these objects have, together, a substantial mass (2–8 times the mass of the present classical Kuiper belt). This suggests that the original solar nebula may have been more extensive and more massive than previously known. Whether the scattered KBOs originated in the Uranus–Neptune zone or the Kuiper-belt region, dynamical studies, constrained by observations of the current population of these objects, could provide valuable new information on the radial distribution of mass in the solar nebula. □

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Experimental evidence for non-exponential decay in quantum tunnelling

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An exponential decay law is the universal hallmark of unstable systems and is observed in all fields of science. This law is not, however, fully consistent with quantum mechanics and deviations from exponential decay have been predicted for short as well as long times^{1–8}. Such deviations have not hitherto been observed experimentally. Here we present experimental evidence for short-time deviation from exponential decay in a quantum tunnelling experiment. Our system consists of ultra-cold sodium atoms that are trapped in an accelerating periodic optical potential created by a standing wave of light. Atoms can escape the wells by quantum tunnelling, and the number that remain can be measured as a function of interaction time for a fixed value of the well depth and acceleration. We observe that for short times the survival probability is initially constant before developing the characteristics of exponential decay. The conceptual simplicity of the experiment enables a detailed comparison with theoretical predictions.

We consider the motion of ultra-cold atoms in an accelerating optical potential of the form $V_0 \cos[2k_L x - k_L a t^2]$, where a is the acceleration, V_0 is the well depth, x is position in the laboratory frame, t is time and k_L is the laser wavenumber^{9,10}. In the accelerating reference frame (x'), this potential becomes $V_0 \cos(2k_L x') + Max'$ where M is the mass of the atom. The linear term in x' leads to an asymmetry in the potential wells. This 'washboard potential' is the same as for the condensed-matter system of an electron moving in a periodic lattice with a d.c. electric field. The periodic optical potential is created by the spatially varying field of a standing wave of light formed by two counter-propagating beams from a single-mode laser. The laser is tuned sufficiently far from atomic resonance so that spontaneous scattering can be neglected, and the atom remains in the ground state. The standing wave is accelerated by ramping the frequency difference of its two beams.

In our previous work on quantum transport of atoms in an

accelerating potential, we performed a spectroscopic study of the band structure and observed Wannier–Stark ladder resonances¹¹. In parallel to our work, Bloch oscillations of ultra-cold atoms were also directly observed¹². Those experiments were done in a regime where tunnelling from the trapped state was negligible. Recently we studied the tunnelling process for larger accelerations, and showed that it is the ultimate limit for this atom accelerator¹³. The trapped state can be considered an unstable quantum system that decays into a reservoir. This can be seen in the 'washboard potential' of the co-moving frame: an atom, trapped in one of the wells, escapes via tunnelling to the continuum. The predicted deviation from exponential decay for short times is related to the fact that the coupling between the system and reservoir is still reversible during that stage. Moreover, the decayed and undecayed state are not yet resolvable, even in principle. In the present system, it is not possible to tell whether an atom is still tracking the accelerating lattice for short times; only in longer times can this question be answered. By then, the decay is irreversible, leading to an exponential decay law. A significant improvement in the signal-to-noise ratio of the data has now enabled us to resolve the short-time tunnelling probability and to observe this phenomenon.

To trap and accelerate a significant number of atoms in a weak potential requires the initial source of atoms to be ultra-cold. We achieve this using a magneto-optic trap consisting of six intersecting

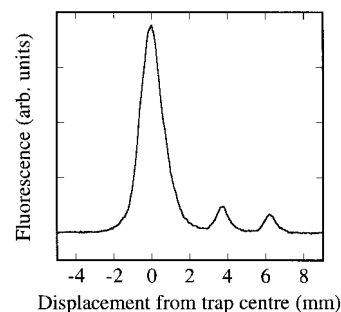


Figure 1 Distribution of atoms after exposure to a three-stage accelerating standing wave for $V_0/\hbar = 80$ kHz. A small acceleration of $1,500 \text{ m s}^{-2}$ was first imposed to trap atoms and separate them from the rest of the distribution. A large acceleration of $7,000 \text{ m s}^{-2}$ was then turned on for a duration of $34 \mu\text{s}$. During this stage atoms tunnel from the trapped state, and are lost to the accelerating potential. Finally the same small acceleration was imposed to separate the atoms that have survived from those that have tunneled out. The duration of the three-step process is 1.5 ms , and the total switching time between the three stages is under 500 ns . A free drift of 3 ms allowed the surviving atoms to separate spatially from the main distribution.

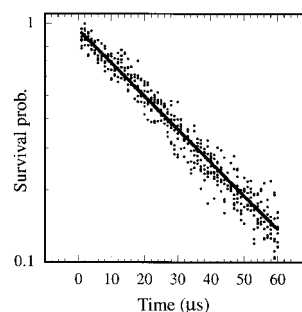


Figure 2 Typical example of experimentally measured survival probability for a small acceleration of $1,200 \text{ m s}^{-2}$, a large acceleration of $4,500 \text{ m s}^{-2}$, and $V_0/\hbar = 50$ kHz as a function of the duration of the large acceleration. Note that the vertical axis is logarithmic. The solid line is a fit to an exponential.

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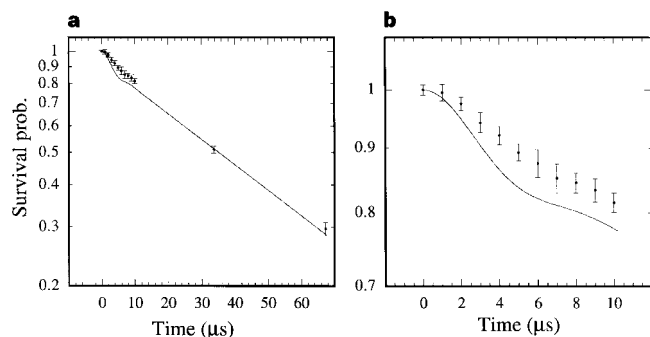


Figure 3 Survival probability as a function of duration of the large acceleration. The experimental data are shown with error bars (one standard deviation). The solid line is the theoretical prediction. For these data the acceleration was $7,000 \text{ m s}^{-2}$, and the potential was $V_0/\hbar = 85 \pm 8.5 \text{ kHz}$. The theoretical calculation used the same acceleration, but the potential was taken as $V_0/\hbar = 74 \text{ kHz}$.

laser beams superimposed on a magnetic field gradient to trap and cool sodium atoms¹⁴. With this configuration, we trap $\sim 10^5$ atoms within a gaussian distribution of position ($\sigma = 0.17 \text{ mm}$) and momentum ($\sigma = 4.6 \hbar/k_1$). The details of our experimental set-up for trapping and cooling have been described elsewhere¹⁵.

The accelerating standing wave is provided by a separate stabilized single-mode dye laser tuned far (up to 20 GHz) from atomic resonance. The output beam from this laser is split into two beams. Each beam is aligned through an acousto-optic modulator that controls its frequency. Both beams are spatially filtered and aligned on the trapped atoms in a counter-propagating configuration, with a beam waist of 1.9 mm in the centre of the trap. When the two beams are at the same frequency, the resulting standing wave is stationary in the laboratory frame. A linear ramp of 4 MHz in 200 μs creates an accelerating potential of $6,000 \text{ m s}^{-2}$.

To study tunnelling in this system, atoms are first trapped and cooled in the magneto-optic trap during a loading time of 6 s. The trapping beams as well as the magnetic field gradient are then turned off, and the accelerating one-dimensional potential is turned on. A three-step acceleration is implemented to measure the loss due to tunnelling¹³. A small acceleration is first imposed to trap atoms and separate them from the rest of the distribution. A large acceleration is then turned on for a controlled duration τ . During this stage, atoms tunnel from the trapped state and are lost to the accelerating potential. Finally the same small acceleration is imposed to separate the atoms that have survived from those that have tunneled out. Once the final velocity is reached, the potential is turned off, allowing a 3-ms free drift. The six beams of the magneto-optic trap are then turned on in zero magnetic field. Atomic motion is effectively frozen at this stage, and the resonance fluorescence is recorded on a charged-coupled-device (CCD) camera. The resulting two-dimensional images are binned in the direction orthogonal to the standing wave to obtain one-dimensional distributions, as shown in Fig. 1. The large peak on the left is from atoms in the initial distribution that were not trapped, the middle peak is from atoms that tunneled during the second step of large acceleration, and the peak on the right is from atoms that survived the large acceleration. The integrated area of the peak on the right is normalized to the total area contained in the middle and right peaks, in order to determine the fraction of survivors.

We have measured the survival probability as a function of τ and find an exponential decay law, as shown for a particular case in Fig. 2. The uncertainty is due primarily to laser power fluctuations, but we were able to obtain very good fits to an exponential decay law. We

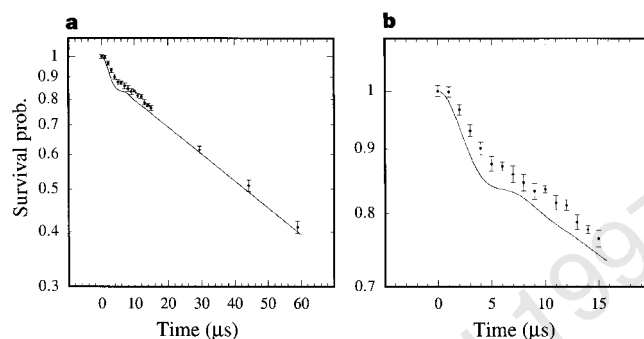


Figure 4 Survival probability as a function of duration of the large acceleration. The experimental data are shown with error bars. The solid line is the theoretical prediction. For these data the acceleration was $9,000 \text{ m s}^{-2}$, and the potential was $V_0/\hbar = 90 \pm 9 \text{ kHz}$. The theoretical calculation used the same acceleration, but the potential was taken as $V_0/\hbar = 92 \text{ kHz}$.

have conducted a detailed study of the decay rates as a function of acceleration and well depth, and find good agreement with a quantum simulation¹³. This process can also be modelled as a Landau-Zener tunnelling process which gives an analytic expression for the tunnelling rate that is in reasonable agreement with experiment.

To observe short-time deviations from the exponential decay, the power of each beam in the standing wave was measured on a photodiode and digitized. In the data analysis we then selected runs for which the power fluctuations were within a $\pm 1\%$ window. The exponential fit was not appreciably altered as the fluctuations were reduced, but the short-time deviation was resolved. Our results are shown in Fig. 3 for an acceleration of $7,000 \text{ m s}^{-2}$ and in Fig. 4 for an acceleration of $9,000 \text{ m s}^{-2}$. Each point represents (on the average) 20 experimental runs, and the error bar is one standard deviation about the mean. Data were taken at intervals of 1 μs for the first 15 μs to obtain the highest resolution in short time. The long-time exponential behaviour was extensively tested in earlier work¹³, and the data shown here for times longer than 15 μs establish the exponents. Figures 3b and 4b show expanded-scale views of the short-time behaviour. The focus of our attention is on timescales shorter than 15 μs . The initial survival probability is flat, owing to a reversible coupling to the bath. The intermediate stage is characterized by a sharper fall-off than the exponential. This can be understood as a 'projection loss' due to a mismatch between trapped states at the two values of the acceleration. Beyond this stage, an irreversible coupling to the reservoir leads to exponential decay.

The theoretical analysis of this problem and comparison with our data proceeds along two directions. The first is a numerical simulation of the time-dependent Schrödinger equation to determine the decay constant for the exponential stage. We find good quantitative agreement with our measurements with no adjustable parameters¹³. To analyse the short-time tunnelling probability and the approach to exponential decay, we use a simple model that provides analytical expressions, and find good agreement with experiment. The starting point for this analysis is the energy structure of a periodic lattice, which consists of energy bands separated by bandgaps. This structure can be represented as a dispersion relation between the energy and quasi-momentum, k . As a familiar point of reference, free-particle motion would be represented as a parabola in this picture as the energy is proportional to the square of the momentum. When an acceleration is imposed, k varies in time as the atom moves through the band. To obtain a simple expression for the tunnelling probability during the stage of large acceleration, we keep only the first bandgap, and

neglect the second and higher gaps. This leads to a modified band structure with a single band, separated by a bandgap from free particle motion. This approximation is valid when the tunnelling rate across the higher gaps is sufficiently high, as is the case in the present work. We treat the non-adiabatic coupling between the trapped and non-trapped states as a weak perturbation, and find that the logarithm of the survival probability in the trapped state is to leading order

$$\ln P(t) = - \int_0^t d\tau (t - \tau) W(\tau) \quad (1)$$

where

$$W(\tau) = \frac{a^2}{2V_0} \int_{-\infty}^{\infty} ds \frac{1}{1 + (s - a\tau/V_0)^2} \frac{1}{1 + s^2} \times \cos \left(\frac{V_0^2}{a} \int_{s - a\tau/V_0}^s \sqrt{1 + z^2} dz \right) \quad (2)$$

In the above expressions, time t is measured in units of $M/4\hbar k_L^2$, the potential amplitude V_0 in $4\hbar k_L^2/M$, and acceleration in $8\hbar k_L^3/M^2$. As $W(\tau)$ decays to zero sufficiently fast for large τ , the long-time behaviour of the survival probability is exponential, $P(t) = \exp(-\alpha t + \beta)$, where α and β are the infinite time integrals of $W(\tau)$ and $\tau W(\tau)$, respectively. In this system there is no long-time deviation from exponential decay, because with a tilted potential the energy spectrum is not bounded from below. On the other hand, $W(\tau)$ is finite for $\tau = 0$, showing that equation (1) is quadratic at short times. Equation (1) is plotted in Figs 3 and 4, and demonstrates good overall agreement with the experimental results. The theory predicts a somewhat larger projection loss than the observed values. This due to the fact that the theory assumes zero acceleration in the first and third stages, creating a larger mismatch between the trapped state in the second stage. We also find that the analytic approximations become better at larger values of the acceleration where Landau–Zener theory is a good model for the tunnelling process.

Here we have reported observed short-time deviation from exponential decay in a tunnelling experiment. In the future we will investigate the possibility of suppressing tunnelling by repeated measurements during the non-exponential time^{6,7}. □

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Chiral discrimination using piezoelectric and optical gas sensors

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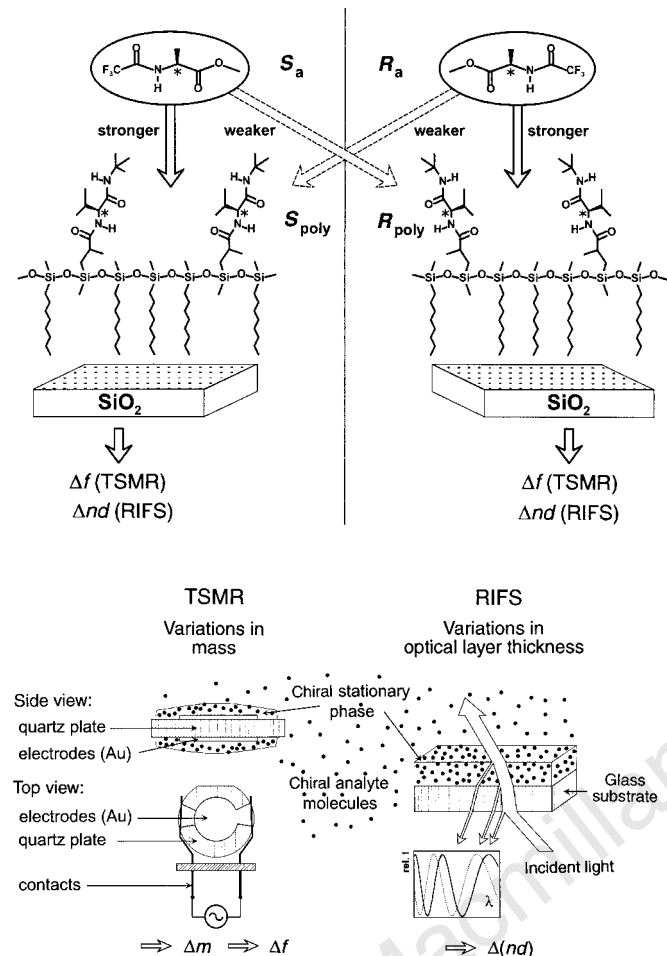
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Odour perception in humans can sometimes discriminate different enantiomers of a chiral compound^{1–3}, such as limonene. Chiral discrimination represents one of the greatest challenges in attempts to devise selective and sensitive gas sensors. The importance of such discrimination for pharmacology is clear, as the physiological effect of enantiomers of drugs and other biologically active molecules may differ significantly⁴. Here we describe two different sensor systems that are capable of recognizing different enantiomers and of qualitatively monitoring the enantiomeric composition of amino-acid derivatives and lactates in the gas phase. One sensor detects changes in mass, owing to binding of the compound being analysed (the 'analyte'), by thickness shear-mode resonance^{5–7}; the other detects changes in the thickness of a surface layer by reflectometric interference spectroscopy^{8–10}. Both devices use the two enantiomers of a chiral polymeric receptor, and offer rapid on-line detection of chiral species with high selectivity.

Natural odour receptors are proteins that use only one enantiomeric form (the L form) of the amino-acid building blocks^{11,12}. In contrast, our sensors apply both enantiomers of a receptor simultaneously. We use the chiral model receptor octyl-Chirasil-Val (Fig. 1), which is derived from the well-known chiral chromatographic stationary phase Chirasil-Val¹³. It contains chiral peptide residues for enantiomer recognition and non-chiral lipophilic side chains¹⁴. Solutions of these polymers were sprayed onto the quartz plates (by airbrush)⁷ or spin-cast on the optical devices¹⁰. The layer thickness ranged between 100 and 300 nm. In addition to the two sets of chiral sensors, reference devices coated with the non-enantioselective polymer poly(dimethylsiloxane) (SE-30) were included in the sensor arrays to recognize artefacts caused by fluctuating gas-phase concentrations or contaminations of the (S)- and (R)-analytes.

Signal transduction (chemical to electrical) was achieved using thickness shear-mode resonators (TSMRs; fundamental frequency, 30 MHz) and reflectometric interference spectroscopy (RIFS) techniques (Fig. 2). When the polymer-coated TSMRs are exposed to analyte gas, sorption of molecules by the polymer generates a change of the oscillating mass which causes a shift of the operating frequency^{5–7}. The sorption strength depends on the interaction mechanisms (H-bridge bonds, dispersion forces) between matrix and analyte molecules. Reflectometric interference spectroscopy was applied to characterize changes in the optical thickness $\Delta(nd)$, where n is refractive index, and d is layer thickness of the polymer layer (swelling on analyte sorption). Interferograms produced by the reflection of light at the multi-layer system glass/polymer/air (Fig. 2) were evaluated^{8–10}.

All the sensors were mounted in the same flow-through cell and simultaneously exposed to analyte gas at a constant temperature of 303 K. The chiral analytes included both enantiomers of *N*-trifluoroacetyl-alanine methyl ester (*N*-TFA-Ala-OMe, ref. 15, Figs 1 and 3), ethyl lactate and methyl lactate (Fig. 4). The test vapours were generated by thermally controlled vaporizers using synthetic air as carrier gas. Typical experiments consisted of



Gas phase:
chiral analyte
(N-TFA-Ala-OMe)

sorption

Polymer phase:
enantioselective
sensor coating
(Octyl-Chirasil-Val)

Transducer

Signal

Figure 1 The principle of chiral recognition by gas sensors: chiral discrimination by preferential sorption of the enantiomers of *N*-trifluoroacetyl-alanine methyl ester (*N*-TFA-Ala-OMe) into enantioselective (*R*)- and (*S*)-octyl-Chirasil-Val polymers. The chemical information is transformed into optical or electrical signals by the respective transducers. TSMR, thickness shear-mode resonator; f , frequency; RIFS, reflectometric interference spectroscopy; n , refractive index; d , layer thickness.

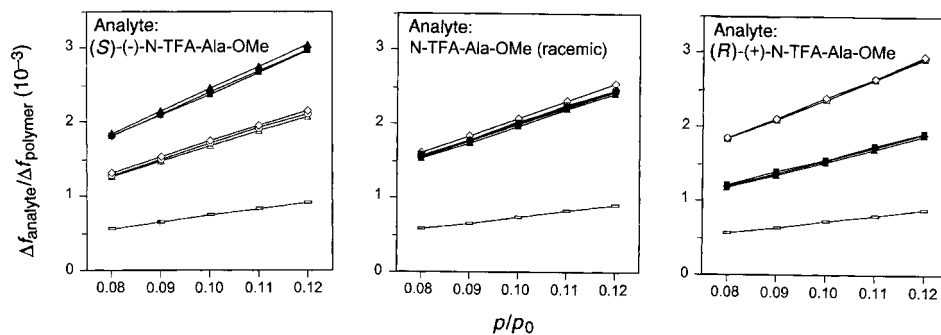
Figure 2 Signal transduction techniques. Left, thickness shear-mode resonators (TSMRs) are mass-sensitive transducers, commonly used for monitoring volatile organic compounds using polymer layers as sensitive coatings. The set-up consists of discrete TSMRs operating at a fundamental frequency of 30 MHz. As shown by Sauerbrey²³, the vibrating frequency of a TSMR changes, to a first approximation, in proportion to the mass deposited onto or removed from the surface: $\Delta f = -Cf_0^2\Delta m/A$, where Δf is the frequency shift in Hz due to the added mass, C is a constant, f_0 is the fundamental frequency of the quartz crystal in Hz, and $\Delta m/A$ is the surface mass loading in g cm^{-2} . Right, Reflectometric interference spectroscopy (RIFS) is based on interference effects in thin transparent films. A light beam passing through a transparent thin polymer film is reflected in part at each of the interfaces (air/polymer and polymer/support). As the two reflected beams travel different optical paths, a phase difference is introduced. If the film thickness d is of the order of a few μm , the difference in optical paths is minute and, even for coherent white light sources, interference effects can be observed. By absorbing analyte molecules in the thin polymer film, the thickness of the film is slightly changed. Bottom right, thin film interference leads to periodic modulation of reflectance with wavelength. Changes in film thickness shift this pattern on the wavelength scale. Consequently, changes in polymer film thickness upon analyte sorption can be determined from the interference spectra with high resolution.

repeated alternating exposures (20 minutes) to air and entrained vapour. The absolute sensor responses are the signal differences between analyte equilibration and purging state (dry air).

Achieving chiral discrimination with gas sensors is fundamentally more difficult than with gas chromatography (GC), because the former uses only one 'theoretical plate' (one absorption/desorption step), whereas in GC discrimination typically results from the cumulative effect of thousands of successive absorption/desorption equilibria^{16–19}. Gas chromatography, however, is an off-line procedure (the same holds for colorimetry²⁰ and fluorescence methods²¹), whereas gas sensors offer the advantage of fast and continuous monitoring. Thus one of the principal goals was to unambiguously demonstrate the ability of sensor arrays to discriminate optical isomers. In contrast to the cyclodextrin derivatives investigated by Ide *et al.*²², the availability of both optical antipodes of the amide polymer ((*R*)- and (*S*)-octyl-Chirasil-Val) allows the possibility of 'cross checks'. Both receptor enantiomers are simultaneously exposed to the analyte enantiomer, so a higher and a lower sensor signal are observed at the same time. In switching from one enantiomer of the analyte to the other, a cross-wise inversion of the signals provided by the two chiral polymers is expected. Artefacts or problems in vaporization, which might lead to chiral discrimination even by non-chiral coatings (as reported in Table 2 of ref. 22), can be definitely excluded this way. Quantitative measurements become

possible. In addition, we used two completely independent sensor systems and gas mixing set-ups and achieved full identity of the respective results. In contrast to ref. 22, these results are in agreement with GC measurements.

The first analyte was *N*-TFA-Ala-OMe ($\Delta\Delta G^0 = 1,050 \text{ J mol}^{-1}$ and $\alpha = 1.57$), where α is the ratio of the net retention times of the respective enantiomers and $\Delta\Delta G^0$ is the enantiomeric difference in Gibbs free energy of sorption¹⁸, where the acid group is protected by esterification and the amino group by a trifluoroacetyl group. In comparative GC experiments, it has been established¹⁴ that the interactions between (*S*)-analyte and (*S*)-polymer as well as (*R*)-analyte and (*R*)-polymer are stronger than between the respective (*R*)-/(*S*)-pairs. We now report corresponding results using gas sensors. In Fig. 3 the relative TSMR signals (normalized to the frequency shift associated with the deposition of the coating) are given for varied analyte gas concentrations. Three (*S*)-polymer and three (*R*)-polymer sensors with different layer thicknesses were chosen. The mass uptake of the (*S*)-sensors (represented by frequency shifts of TSMRs) upon exposure to (*S*)-*N*-TFA-Ala-OMe is significantly larger than that of the (*R*)-sensors. For the (*R*)-analyte, the expected cross-wise inversion is experimentally observed: stronger sorption into the (*R*)-polymer occurs. As expected, the signals of the chiral sensors upon exposure to the racemate are identical and have values intermediate between those of the (*S*)-analyte



Coating:	Polydimethylsiloxane	(S)-Octyl-Chirasil-Val	(R)-Octyl-Chirasil-Val
Sensor index	SE-30	S2 S4 S5	R1 R2 R4

Figure 3 Normalized (with regard to the frequency shift due to polymer deposition) TSMR responses to different concentrations (p/p_0 : adjusted pressure with respect to the saturation vapour pressure at 298K) of (R)-, (S)- and racemic *N*-trifluoroacetyl-alanine methyl ester. The responses of three (R)- (open symbols), three (S)-sensors (filled symbols) and one SE-30 sensor are displayed. Sensor index is given at the bottom.

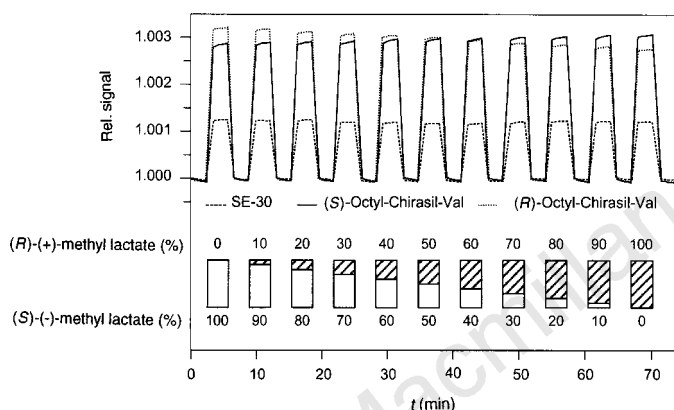


Figure 4 Top, relative RIFS-sensor signals (normalized with regard to the layer thickness without analyte gas, as described in the text of two chiral sensors ((S)-sensor, solid line; (R)-sensor, dotted line) and an additional SE-30-sensor (dashed line) upon exposure to different enantiomeric compositions of methyl lactate. The relative fraction of (R)- and (S)-enantiomer of methyl lactate is indicated at the bottom (hatched boxes).

((R)-analyte)/(S)-sensor pairs and the (S)-analyte ((R)-analyte)/(R)-sensor pairs. In addition to the signals of the enantioselective polymers, the responses of one of the non-chiral sensors (SE-30) are shown in Fig. 3. As their responses are proportional to concentration regardless of which enantiomer is sorbed by the polymer, their signals are displayed as an indication of the analyte gas concentrations. Similar results were achieved for the lactates.

Additional experiments (Fig. 4) demonstrate the ability to determine quantitatively the enantiomeric composition of various mixtures of *N*-TFA-Ala-OMe (or the lactates), exploiting the enhanced resolution of the simultaneous application of both receptor enantiomers. We chose methyl lactate ($\Delta\Delta G^0 = 285 \text{ J mol}^{-1}$, $\alpha = 1.13$), where $\Delta\Delta G^0$ is considerably lower than for *N*-TFA-Ala-OMe and closer to most practical cases. Nonetheless, it was possible to determine the enantiomeric composition from the raw TSMR- or RIFS-sensor signals (Fig. 4) without mathematical treatment of the data. As an example, the relative sensor signals (change in optical layer thickness on analyte exposure normalized to the layer thickness in the absence of analyte gas) are shown in Fig. 4. The maximum change in optical thickness was $\sim 3 \times 10^{-3}$ times the total polymer layer thickness. The response of the SE-30 sensor was identical for the 11 sequential exposures, indicating the precision of the adjusted gas flows and serving as a reference for the chiral sensors. The chiral sensors (roughly equal layer thickness) showed systematic and consistent signal increases or decreases with changing enantiomeric composition of the analyte. The signal of the (R)-sensor increased with increasing (S)-analyte concentration, and vice versa for the (S)-sensor. A 10% change in composition is easily recognized: such accuracy cannot be achieved by olfaction. The signals of the two chiral sensors match for the racemic mixture. This is consistent with

GC results: lower affinity occurs between (S)-polymer/(S)-analyte compared to (R)-polymer/(S)-analyte.

Both sensor systems presented here can be used quite effectively to discriminate between the (R)- and (S)-enantiomers of selected gaseous species, providing 10% resolution ($\Delta\Delta G^0 = 30 \text{ J mol}^{-1}$ at $\alpha = 1.13$) for a fast, real-time, *in situ* determination of enantiomeric excess. Such sensors may prove useful in controlling the enantiomeric purity of anaesthetics and drugs in pharmacology, or in monitoring the quality of, for example, lactates, which are synthesized in industrial quantities. □

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A biosensor that uses ion-channel switches

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Biosensors are molecular sensors that combine a biological recognition mechanism with a physical transduction technique. They provide a new class of inexpensive, portable instrument that permit sophisticated analytical measurements to be undertaken rapidly at decentralized locations¹. However, the adoption of biosensors for practical applications other than the measurement of blood glucose is currently limited by the expense, insensitivity and inflexibility of the available transduction methods. Here we describe the development of a biosensing technique in which the conductance of a population of molecular ion channels is switched by the recognition event. The approach mimics biological sensory functions^{2,3} and can be used with most types of receptor, including antibodies and nucleotides. The technique is very flexible and even in its simplest form it is sensitive to picomolar concentrations of proteins. The sensor is essentially an impedance element whose dimensions can readily be reduced to become an integral component of a microelectronic circuit. It may be used in a wide range of applications and in complex media, including blood. These uses might include cell typing, the detection of large proteins, viruses, antibodies, DNA, electrolytes, drugs, pesticides and other low-molecular-weight compounds.

The active elements of the ion-channel switch comprise a gold electrode to which is tethered a lipid membrane containing gramicidin ion channels⁴ linked to antibodies. The molecular structure of the tethered membrane^{5–14} results in an ionic reservoir¹⁵ being formed between the gold electrode and the membrane. The ionic reservoir can be accessed electrically through connection to the gold electrode. In the presence of an applied potential, ions flow between the reservoir and the external solution when the channels are conductive. The ion current is switched off when mobile channels diffusing within the outer half of the membrane become crosslinked to antibodies immobilized at the membrane surface. This prevents them forming dimers with channels immobilized within the inner half of the membrane. The number of dimers is measured from the

electrical conduction of the membrane. The switch has a high gain; a single channel facilitates the flux of up to a million ions per second. A quantitative model of the biosensor has been verified experimentally; see Supplementary Information.

The detection of analytes possessing multiple recognition sites is performed using the structure shown schematically in Fig. 1a. This structure is assembled using a combination of sulphur–gold chemistry and physisorption as described in Fig. 1 legend. The membrane consists of lipids and channels, some immobilized on the gold surface and some diffusing laterally within the plane of the membrane. The antibodies on the mobile channels scan an area of the order of $1\ \mu\text{m}^2$ in less than 5 minutes. Thus with a low density of channels and a high density of immobilized antibodies, each channel can access up to 10^3 more capture antibodies than if the gating mechanism were triggered by a directing binding of analyte to the channels. The speed and sensitivity of the biosensor response may be adjusted in direct proportion to the number of binding sites accessible to each mobile channel. This allows for quantitative detection of analyte from sub-picomolar to micromolar concentrations in less than 10 minutes.

The immobilized membrane components are shown in Fig. 2a. These compounds possess common sulphur and reservoir-forming segments. A fraction of the tethered species are hydrophilic spacer molecules, provided to increase the volume within the reservoir and to improve the conductivity of the membrane. Tethered membrane-spanning lipids are included to improve the stability of the membrane. This is reminiscent of the role played by membrane-spanning lipids found in archaeobacteria¹⁶. These bacteria can survive at temperatures in excess of 100°C . Tethering a substantial fraction of the lipid both stabilizes the lamella phase necessary for the formation of the membrane and inhibits the insertion of further material following the membrane assembly. Tethered membranes formed in this manner are stable over many months. A fraction of the membrane-spanning lipid is coupled to antibodies. This is the immobilized population of antibodies necessary for the switch function. The mobile membrane-forming species are shown in Fig. 2b.

A competitive version of the switch has been assembled and demonstrated for the detection of small analytes with only a single epitopic site. This is shown schematically in Fig. 1b.

Figure 2 Components used in the ICS biosensor assembly. **a**, The immobilized components of the membrane consist of a mixture of tethered gramicidin, gAYYSSB_n (G_n), double-length reservoir half-membrane-spanning phytanyl lipids, (DLP), and full-membrane-spanning lipids, (MSL). The surface density of these tethered species is controlled by dilution with a low-molecular-weight hydrophilic spacer, mercaptoacetic acid disulphide MAAD or ethylene disulphide (EDS). A fraction of the tethered membrane spanning lipid, MSL_n, is biotinylated for subsequent coupling to streptavidin and an antibody fragment. These compounds are based on a common benzyl disulphide attachment moiety and an ethylene glycol chain. The thickness of the reservoir region is determined by the length (~4 nm) of the ethylene glycol chain segment. **b**, The main mobile component is a 30:70 (mole ratio) mix of glycerodiphytanylether (GDPE):diphytanyl ether phosphatidylcholine (DPEPC), which is mixed with a small fraction of mobile ion channels (G_n). For large analyte detection, the channel species employed is gA5XB. The biotinylation site on gA5XB is used for subsequent coupling to streptavidin and an antibody fragment. For small analyte detection, the mobile channel is the hapten-linked gA4Xdig.

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a

gAYYSSBn (G_T) GRAMICIDIN

DLP

MAAD

EDS

MSL

MSL_α

b

GDPE R= OH

DPEPC R= $-\text{O}-\text{P}(=\text{O})(\text{O}^-)-\text{O}-\text{CH}_2-\text{CH}_2-\text{N}^+(\text{CH}_3)_3$

gA5XB (G_{α})

GRAMICIDIN' $\text{O}-\text{C}(=\text{O})-(\text{CH}_2)_6-\text{NH}-\text{C}(=\text{O})-(\text{CH}_2)_6-\text{NH}$ (4) $\text{NH}-\text{C}(=\text{O})-(\text{CH}_2)_6-\text{NH}-\text{C}(=\text{O})-\text{S}-\text{C}_4\text{H}_3\text{N}_2\text{O}_2$

gA4XDig (G_{α})

GRAMICIDIN' $\text{O}-\text{C}(=\text{O})-(\text{CH}_2)_6-\text{NH}-\text{C}(=\text{O})-(\text{CH}_2)_6-\text{NH}-\text{C}(=\text{O})-\text{C}_{27}\text{H}_{45}\text{O}_5$

The ion-channel switch (ICS) biosensor requires none of the washing or reagent additions of the enzyme-linked immunosorbent assay (ELISA). This is a consequence of the capture and reporter antibodies being preassembled at the membrane surface. From the phase relationship between the excitation potential and the current flow we derive the admittance at minimum phase, $Y_{\phi_{\min}}$. For large, multiply bound analytes the sensor response is determined from the maximum rate of change of admittance (see Supplementary Information), $-(dY_{\phi_{\min}}/dt)_{\max}$, which can be shown by numerical simulation to be a linear function of the concentration of analyte over more than six decades of detectable admittance change. The maximum slope can typically be attained after only 10% change in $Y_{\phi_{\min}}$ and provides ~ 3 –4 decades of dynamic range for each 2 decades of variation of the initial $Y_{\phi_{\min}}$.

The response of the ICS biosensor to large analytes may be demonstrated using thyroid-stimulating hormone, TSH (relative molecular mass 28,000, M_r 28K). TSH comprises an α and β subunit to which it is possible to bind complementary Fabs (antigen-binding fragments of an antibody). To switch off the ion

channels through crosslinking, a matched pair of antibodies is used, each one sensitive to one of the non-overlapping binding sites on the TSH. The response is shown in Fig. 3. If the membrane surface is prepared exclusively with biotinylated anti-ferritin Fab', anti- α -TSH Fab' or anti- β -TSH Fab', the addition of TSH results in a small or negligible change in admittance. If however, an equal mixture of biotinylated anti- α -TSH and anti- β -TSH Fab' is bound, the addition of TSH elicits a dramatic reduction in membrane admittance. The dependence of the admittance response on the Fab' composition is consistent with the proposed mechanism. All of the six differently targeted, complementary anti-TSH Fab' fragments we have tested have functioned successfully in the ICS biosensor. Digoxin (M_r 781), demonstrates the response of the ICS biosensor to small analytes. The mobile channels carry a digoxin hapten as shown in Fig. 2b. In the absence of analyte, the mobile channels crosslink to the immobilized antibody fragments preventing the formation of dimers and turning off the membrane conductance. The introduction of analyte competes with the hapten for the binding site within the antibody fragment, liberating the channel

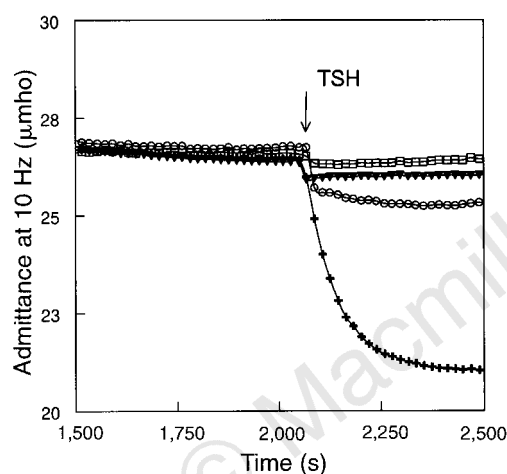


Figure 3 Response of ICS biosensor to TSH. **a**, Assembly procedures. First, a tethered membrane 0.16 mm² in area is formed on a freshly evaporated gold film. The gold film is 100 nm thick and is bonded to a clean glass microscope slide using a 5-nm layer of chromium. The slide is then immersed in an ethanol solution of the immobile lipid-layer mixture for 1 h at 20 °C. The mixture comprises 59 μM DLP, 4.4 nM MSL-4XB, 4.4 μM MSLOH, 7.5 nM gAYSSB_n, and 35 μM EDS (see Fig. 2 legend for abbreviations). The slide is then removed and rinsed thoroughly in ethanol, air-dried, and clamped to a series of teflon cylinders, each 4 mm internal diameter, 5 mm long, each defining a well volume of 200 μl above the coated gold surface. A further 5 μl of mobile-layer mixture in ethanol solution is added, which contains 350 nM gA-5XB and a 14 mM (30:70) mix of GDPE:DPEPC, after which the slide is rinsed with ~500 μl of phosphate buffered saline (PBS). This induces the spontaneous formation of a bilayer membrane. Care is taken not to introduce air bubbles or to take the newly formed membrane surface through an air-water interface. Having rinsed three times with PBS to eliminate any residual excess membrane lipid, 5 μl of 1.6 μM streptavidin in PBS is added. Following incubation for 10 min, the supernatant is thoroughly rinsed with PBS and a further 20 μl of 0.2 μM b-Fab' is added. This is then incubated for a further 10 min and thoroughly rinsed with PBS to remove residual material. The sensor is now stored ready to use. **b**, Measurement procedure. The admittance is measured at 10 Hz using an excitation amplitude of 50 mV and an offset potential of -300 mV at the gold electrode relative to the test solution. Traces are shown using different specificities of biotinylated Fab': open squares, b-antiferritin; filled triangles, b-anti- α -TSH; circles, b-anti- β -TSH; and crosses, a 50:50 mix of b-anti- α -TSH and b-anti- β -TSH. 2 nM of TSH is added at the arrow. The mixed population of biotinylated anti- α and biotinylated anti- β Fab' elicited a substantial response when the TSH was added. However, the other three biotinylated Fab' populations only elicited a small or negligible response when used in isolation.

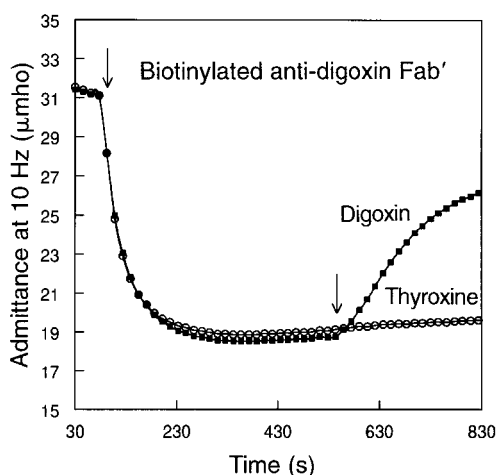


Figure 4 Response to digoxin. A similar membrane is prepared to that described in Fig. 3 legend. The principal difference is that the concentration of MSL_α is increased tenfold and G_α is replaced by G_h. The total concentration of MSL is maintained. Following the formation of the membrane, it is incubated with 40 nM streptavidin for 10 min and rinsed again. The addition of 5 μl of 50 nM biotinylated-antidigoxin (first arrow) results in a decrease in the admittance. The membrane is then thoroughly rinsed with PBS and stored ready for use. The addition of 3 μM (5 μl) digoxin (second arrow) causes the admittance to increase (filled squares). The rate and size of this increase is related to the concentration of digoxin. The process may be repeated many times following rinsing with PBS solution. A control is shown in which thyroxine was used in place of digoxin, eliciting no response (circles).

and increasing the conductance. An example of the formation of channel–Fab' crosslinked species and their liberation upon the addition of digoxin is shown in Fig. 4. A control is also shown in which the addition of thyroxine fails to elicit a response. The absence of biotins on the lipid tether, the absence of the streptavidin linker or the correctly targeted Fab' eliminates the response. A quantitative measure of analyte concentration may be obtained from either the absolute initial rate of admittance increase, or the equilibrium fractional admittance change (see Supplementary Information). These are essentially proportional to each other over the sensitivity range of the competitive assay. The range may be adjusted by varying the immobilised Fab' surface density.

By varying the nature and type of receptor in the ICS biosensor, we have applied the technique to blood typing, the detection of bacteria, virus particles, DNA, drugs, antibodies and electrolytes. Receptors include antibodies, enzymes, DNA, binding proteins and synthetic ligands. The ion-transport properties of channels and ionophores other than gramicidin have also been studied^{17,18}. For example, we have incorporated the K⁺ ionophore valinomycin into a tethered membrane and successfully measured potassium concentration over physiologically relevant concentrations. The biosensor functions in human serum, plasma and whole blood. The biosensor may be assembled and measured on electrodes of dimensions from square centimetres to square micrometres. As the membrane area is decreased, the membrane leakage conductance decreases proportionally, but the conductance per channel remains constant. This means that with small electrodes (<30 µm diameter) it becomes possible to resolve the current transients associated with individual channels. Operation of the biosensor under these conditions of low channel density, coupled with the use of multi-electrode arrays of such membranes, promises to increase significantly the sensitivity of the device. □

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Supplementary Information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from Mary Sheehan at the London editorial office of Nature.

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Synthesis and X-ray structure of dumb-bell-shaped C₁₂₀

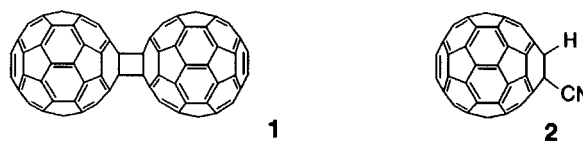
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The discovery and large-scale synthesis of fullerenes have aroused interdisciplinary interest in these closed-cage molecules^{1–6}. C₆₀ can be photopolymerized into a form in which the cages are thought to be linked by cyclic C₄ units in a [2 + 2] cycloaddition⁷, provoking theoretical studies of the C₆₀ dimer^{8–15}, the smallest subunit of such a polymer. The C₆₀ dimers C₁₂₀O (refs 16, 17), C₁₂₁H₂ (ref. 17) and C₁₂₀O₂ (ref. 18) have been reported, in which the two C₆₀ molecules are linked by, respectively, a furan group, a cyclopentane ring and a cyclobutane ring plus two oxygen bridges; but the simplest dimer, C₁₂₀ linked by a cyclobutane ring alone, has not so far been observed. We now report that this dumb-bell-shaped molecule can be synthesized by a solid-state mechanochemical reaction of C₆₀ with potassium cyanide. Our X-ray structural analysis shows that the C₄ ring connecting the cages is square rather than rectangular—the latter is predicted theoretically^{8,9,13–15}. The dimer dissociates cleanly into two C₆₀ molecules on heating or one-electron reduction, but in the gas phase during mass-spectrometric measurements it undergoes successive loss of C₂ units, shrinking to even-numbered fullerenes such as C₁₁₈ and C₁₁₆ in a sequence similar to that seen for other large fullerenes^{19,20}.

Recently, we developed a new method to derivatize C₆₀ in the solid state by the use of a 'vibrating mill'²¹. The key feature of this method is a high-speed vibration technique, which can activate the reaction system by bringing the reagents into very close contact at the preparative scale and by providing extra mechanical energy, much more effectively than the ball-milling technique²². The reaction of C₆₀ with KCN in the solid state has led to the first preparation of dumb-bell-shaped C₁₂₀ (1) instead of formation of C₆₀H(CN) (2)²³ after quenching with trifluoroacetic acid.



A mixture of C₆₀ and 20 molar equivalents of KCN powder was vigorously vibrated for 30 min under nitrogen according to our previous procedure²¹. Analysis by high-performance liquid chromatography of the reaction mixture dissolved in *o*-dichlorobenzene (ODCB) on a Cosmosil Buckyprep column with toluene as the eluent showed only one major product besides unchanged C₆₀. Separation by flash chromatography on silica gel, eluted with hexane–toluene and then with toluene–ODCB, gave 70% of recovered C₆₀ and 18% of C₁₂₀ (1). Its structure was unequivocally determined as the [2 + 2] adduct of C₆₀ based on the evidence shown below.

The product, isolated as a dark brown powder, has very low solubility in CS₂ and toluene, but is reasonably soluble (1–2 mg ml^{−1}) in ODCB. The ¹³C NMR spectrum (Fig. 1) exhibited 15 signals (including one overlapped signal) in the *sp*² region and one signal at 76.22 p.p.m. in the *sp*³ region, which are fully consistent with the assigned structure with D_{2h} symmetry. A comparison of the present data with the ¹³C magic-angle spinning (MAS) NMR spectra of C₆₀ polymers prepared under high pressure^{24,25} has

established that the dumb-bell-shaped C_{120} is indeed the essential subunit of these polymers.

The Fourier-transform infrared spectrum of **1** showed more infrared-active peaks than C_{60} and resembled that of polymer prepared by photo-irradiation⁷, thus giving a strong support for the presence of the [2 + 2] structure in the photoproduct polymer. Just like other 1,2-dihydro[60]fullerenes, the colour of **1** in toluene and in ODCB is brown. The ultraviolet-visible spectrum of **1** in toluene exhibited absorptions at 328, 434 and 698 nm, which are typical for 1,2-dihydrofullerene derivatives. The elemental analysis of **1** showed complete absence of any hydrogen or nitrogen.

Several mass-spectral techniques including fast atom bombardment (FAB), atmospheric pressure chemical ionization (APCI) and

electrospray ionization (ESI) in both positive- and negative-ion modes did not show any molecular ion peak of **1** but a strong peak for C_{60} . Only a weak peak at mass/charge m/z 1,441 (M^+) together with a series of peaks corresponding to the loss of C_{2n} ($n = 1-5$) from C_{120} and a base peak at m/z 720 for C_{60} were observed in both Fourier-transform ion cyclotron resonance (FTICR) (Fig. 2) and matrix-assisted laser desorption-ionization (MALDI) time-of-flight mass spectra. This intriguing result indicates that C_{120} not only dissociates into C_{60} but also reduces its size by extruding C_2 units and rearranging into even-numbered fullerenes C_{118} , C_{116} and so on^{19,20}.

The structure of **1** was determined by X-ray crystallography for the single crystal grown in an ODCB solution. As shown in Fig. 3, dimer **1**

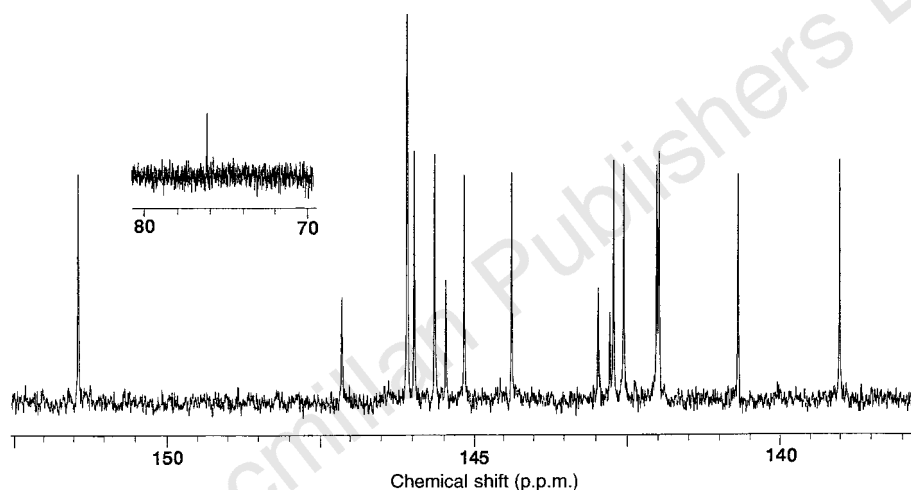


Figure 1 The ^{13}C NMR (150.8 MHz) spectrum of fullerene dimer **1** in $\text{ODCB-}d_4$: chemical shift δ (p.p.m.): 151.42 (8C), 147.14 (4C), 146.08 (8C $\times$ 2), 145.97 (8C), 145.65 (8C), 145.48 (4C), 145.18 (8C), 144.37 (8C), 142.97 (4C), 142.73 (8C), 142.56 (8C), 142.02 (8C), 141.99 (8C), 140.70 (8C), 139.02 (8C), 76.22 (4C). The signal at δ 142.79 p.p.m. is that of C_{60} .

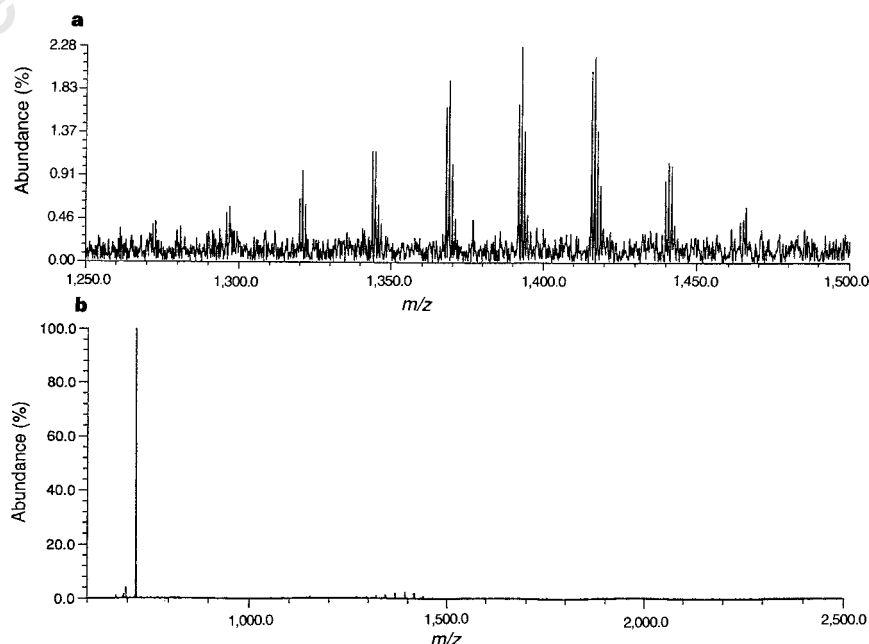


Figure 2 The positive-ion Fourier-transform ion cyclotron resonance (FTICR) mass spectra of fullerene dimer **1**. **a**, An expanded plot showing the molecular ion peak of C_{120} at m/z 1,441 (intensity 1.0%) (plus isotopic peaks) and a series of peaks for $C_{120} - C_{2n}$ ($n = 1-5$), that is, m/z 1,417 (C_{118} , 2.2%), 1,393 (C_{116} , 2.3%), 1,369 (C_{114} , 1.9%), 1,345 (C_{112} , 1.2%) and 1,321 (C_{110} , 1.0%). The spectrum also exhibits a very weak peak at m/z 1,465, which indicates the formation of a small amount of C_{122} by the process of $C_{120} + C_2$. **b**, A full-range spectrum showing a base peak for C_{60} at m/z 720 (intensity 100%) and a cluster of peaks around m/z 1,440.

is composed of two C_{60} cages sharing a cyclobutane ring. Previous theoretical studies predicted the intra-cage bond (C1–C2 in Fig. 3) to be rather long (1.59–1.62 Å)^{8,9,13–15} compared with the inter-cage bond (C1–C1* in Fig. 3). Present results show that there is no great difference in the length of these bonds. The former bond length is 1.581(7) Å, which is rather short compared to reported values for the corresponding bond in substituted 1,2-dihydrofullerenes²⁶. The relative elongation of the inter-cage bond (1.575(7) Å) compared with the ordinary $C(sp^3)$ – $C(sp^3)$ single bond is well reflected in its ready thermal cleavage, as will be discussed below.

Differential scanning calorimetry was used to follow the thermal behaviour of **1** in the range 80–220 °C at a rate of 1 °C min^{–1}. An endothermic peak from 150 to 175 °C and centred at 162 °C was observed in the heating process, whereas no such peak was observed in the cooling scan. Moreover, C_{120} was found to dissociate quantitatively into C_{60} by heating its ODCB solution at 175 °C for 15 min. This decomposition temperature is near to those reported for polymers prepared by photo-irradiation²⁷ and under high pressure^{28,29}.

Cyclic voltammetry of **1** (0.25 mM in ODCB/0.05 M Bu_4NBF_4 ; Pt disk and Pt wire as working and counter electrodes, respectively; scan rate, 20 mV s^{–1}) exhibited three reversible reductions at $E_{1/2}$ – 1.14, – 1.53 and – 1.99 V versus ferrocene/ferrocenium, which are almost the same as those of C_{60} (– 1.12, – 1.52 and – 1.99 V) observed under the same conditions. The only noticeable difference

in the electrochemical behaviour between **1** and C_{60} is the presence of a small shoulder at the first reduction peak of **1** in both cyclic voltammetry and differential pulse voltammetry. Taken together, these results indicate that dimer **1** readily dissociates into the C_{60} anion radical and C_{60} immediately after C_{120} acquires one negative charge.

The very close reduction potentials of C_{120} and C_{60} suggests that C_{120} should have equal chemical reactivity to C_{60} . In fact, the versatile Bingel reaction³⁰ was found to be able to functionalize **1**. Reaction of dimer **1** in ODCB solution with one equivalent each of diethyl bromomalonate and 1,8-diazabicyclo[5.4.0]undec-7-ene for 30 min at room temperature afforded monoadduct of **1** in 44% yield along with 24% of unchanged **1** after separation on JAIGEL – 1H + 2H preparative gel permeation chromatography (GPC) columns eluted with toluene. The adduct was a mixture of at least three positional isomers as judged from HPLC and ¹H NMR data.

The dimerization of C_{60} did not take place without KCN in the solid state. Furthermore, no cyanated product **2**²³ was isolated in the solid-state reaction of C_{60} with KCN. The highly selective synthesis of C_{120} observed here is ascribed to a reaction pathway totally different from that in the liquid phase²³, and to the unique property of cyanide ion behaving both as a nucleophile and as a good leaving group. The solid-state reactions of C_{60} with other nucleophiles did not afford C_{120} (ref. 21). We suppose that the present dimerization

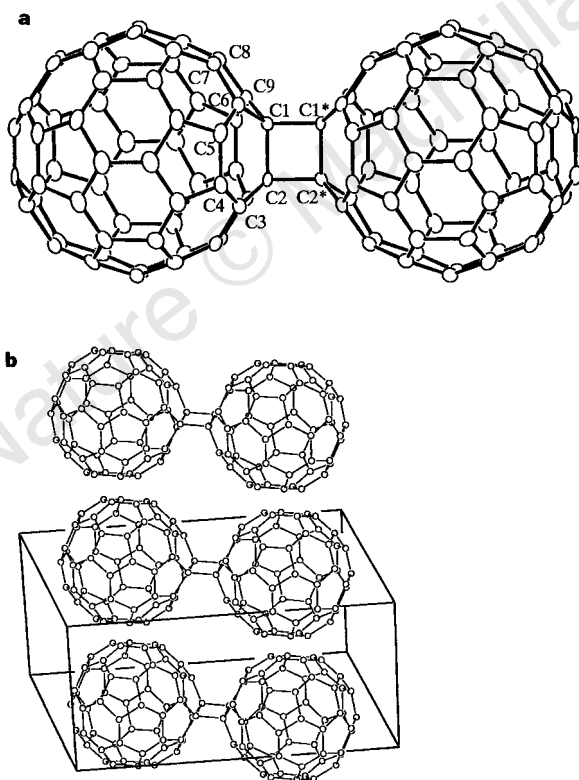


Figure 3 **a**, Structure of fullerene dimer **1**, as determined by X-ray crystallography. Selected bond lengths (Å) and angles (degrees) are: C1–C1*, 1.575(7); C1–C2, 1.581(7); C2–C3, 1.530(8); C3–C4, 1.374(7); C4–C5, 1.468(8); C5–C6, 1.358(9); C6–C1, 1.528(7); C6–C7, 1.445(8); C7–C8, 1.457(9); C2–C1–C6, 115.4(5); C2–C1–C9, 115.2(4); C6–C1–C9, 100.7(4); C2–C1–C1*, 90.3(4); C1–C2–C2*, 89.7(4). **b**, A crystal packing diagram. The R and R_w values were 0.067 and 0.096, respectively. The solvent molecules were contained in a 4:1 ratio in the crystal of **1**, but are omitted from these figures for clarity. Further material characterizing **1**, including X-ray structure details, are available as Supplementary Information.

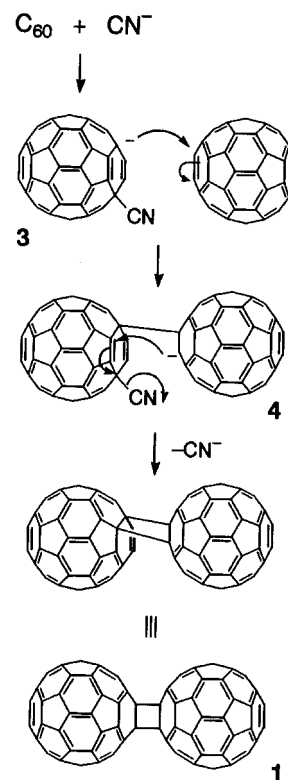


Figure 4 Proposed reaction mechanism for the formation of dimer **1**.

occurs in a similar fashion to the so-called benzoin condensation³¹. As shown in Fig. 4, cyanide ion first adds to C₆₀ to form C₆₀(CN)[−] (3), which can react with the closely located molecule of C₆₀ in the 1,4-addition mode to minimize steric congestion and gives anion 4. Then an intramolecular S_N2' reaction of 4 furnishes the [2 + 2] dimer 1. A further reaction of dimer 1 with cyanide ion could occur, but this would make 1 acquire a negative charge together with a cyano group, and would cause the rupture of its [2 + 2] bonds as inferred from the above-mentioned electrochemical behaviour of 1. Thus the overall reaction results in the highly selective formation of dimer C₁₂₀ instead of oligomers and polymers.

In single crystals of 1 obtained in the present study, the C₁₂₀ molecules are arrayed in highly ordered layers, different from the face-centred cubic arrangement of C₆₀. Photo-irradiation of this crystalline material should lead to the formation of a C₆₀ polymer, which might have a more well-defined structure than previously reported ones^{7,32}. The present result indicates that a thermally forbidden [2 + 2] process of a highly electron-deficient and strained double bond such as that in fullerenes can take place under solid-state reaction conditions catalysed by cyanide ion. Application of the present method to C₇₀ is expected to lead to another new carbon allotrope, C₁₄₀. □

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The Lu–Hf dating of garnets and the ages of the Alpine high-pressure metamorphism

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It remains controversial whether burial and exhumation in mountain belts represent episodic or continuous processes^{1–20}. Regional patterns of crystallization and closure ages of high-pressure rocks may help to discriminate one mode from the other but, unfortunately, metamorphic geochronology suffers from several limitations. Consequently, no consensus exists on the timing of high-pressure metamorphic events, even for the Alps—which have been the subject of two centuries of field work. Here we report lutetium–hafnium (Lu–Hf) mineral ages on eclogites from the Alps as obtained by plasma-source mass spectrometry. We find that the Lu/Hf ratio of garnet is particularly high, which helps to provide precise ages. Eclogites from three adjacent units of the western Alps give (from bottom to top) diachronous Lu–Hf garnet ages of 32.8 ± 1.2, 49.1 ± 1.2 and 69.2 ± 2.7 Myr. These results indicate that the Alpine high-pressure metamorphism did not occur as a single episode some 80–120 Myr ago^{6,7,10,18}, but rather that burial and exhumation represent continuous and relatively recent processes.

With the discovery of the coesite-bearing quartzites of Dora Maira in the western Italian Alps¹, the exhumation history of rocks that had been buried to ~100 km depth during the formation of the Alps provided fresh insight into alpine tectonic processes. Published data, however, emphasize both the extent of inconsistency of the chronological data on high-pressure metamorphism and its far-reaching geodynamic implications^{2–5}. The Eoalpine age (100–120 Myr) inferred from a discordant array of zircons⁶ and from ³⁹Ar–⁴⁰Ar dating in phengites^{7,8} seems to support a widely accepted view that the exhumation of eclogites is coeval with the continuing subduction of the oceanic Tethyan lithosphere under the African plate⁹. Eclogite burial and exhumation also appear to be Eoalpine in age in the Sesia-Lanzo unit with Rb–Sr, K–Ar and U–Pb ages in the range 60–114 Myr (refs 10–12). In contrast, the Late Eocene–Early Oligocene age found for Dora Maira^{13,14}—as a lower concordia intercept defined by zircons and as a U–Pb isochron on ellenbergerite, which was later confirmed by U–Pb ion-probe data on zircons¹⁵—rather suggests that burial and exhumation are far more recent and coeval with the collision between the Apulian and European plates. Sm–Nd and U–Pb Eocene ages are also known from other eclogite localities in the western and central Alps^{16–18}. As these eclogites seem to have been overprinted in the greenschist facies during the Oligocene (25–35 Myr ago) at the latest^{7,19}, the value of the exhumation rate may vary from a fraction of a millimetre to several centimetres per year depending on which age is accepted for the high-pressure metamorphism.

Other than K–Ar, of which the significance is obscured by the

Table 1 Lu–Hf and Sm–Nd data

	Lu (p.p.m.)	Hf (p.p.m.)	$^{176}\text{Lu}/^{177}\text{Hf}$	$^{176}\text{Hf}/^{177}\text{Hf}$	Sm (p.p.m.)	Nd (p.p.m.)	$^{147}\text{Sm}/^{144}\text{Nd}$	$^{143}\text{Nd}/^{144}\text{Nd}$
Sesia (Lillianes-Fontainemore)								
Whole rock	0.211	0.262	0.116	0.282632 (72)	4.62	19.61	0.1422	0.512106 (26)
	0.205	0.221	0.132	0.282877 (95)	4.61	19.53	0.1424	0.512178 (27)
	0.197	0.267	0.105	0.282769 (32)	4.64	19.57	0.1428	0.512093 (24)
Garnet	1.02	0.0390	3.69	0.287667 (74)	0.203	0.253	0.4840	0.510662 (52)
Garnet (impure)	0.833	0.0511	2.31	0.286051 (84)	0.619	1.96	0.1906	0.512003 (23)
Clinopyroxene	0.0128	0.185	0.0798	0.282535 (42)	2.21	8.43	0.1581	0.513154 (27)
Phengite	0.00315	0.0239	0.0187	0.282731 (151)	3.33	7.60	0.2643	0.513198 (22)
Monviso (Lago Superiore)								
Whole rock	0.643	0.454	0.192	0.283234 (32)	7.55	25.17	0.1807	0.513012 (22)
Garnet	1.75	0.0311	8.00	0.290696 (91)			0.65	0.513131 (24)
Clinopyroxene					3.80	12.58	0.1823	0.513041 (21)
Dora-Maira (Parigi)								
Whole rock	0.267	0.144	0.263	0.282828 (22)	8.55	41.96	0.1228	0.512189 (18)
Garnet	1.67	0.107	2.21	0.284067 (28)			0.089	0.511738 (31)

The replicates of Sesia-Lanzo whole rocks represent different powder aliquots. The numbers in parentheses are twice the standard error. The Nd and Hf isotopic compositions of the Monviso whole rock sample plots in the field of mid-ocean-ridge basalts, whereas those of the Dora Maira massif and the Sesia-Lanzo zone plot in the field of continental rocks.

unpredictable presence of excess argon, high-temperature paths of eclogite exhumation have so far been dated using only two methods. U–Pb dating of zircons gives, in principle, reliable crystallization ages, although zircon is not a mineral specific to high-pressure assemblages. In addition, as best shown in the case of Dora Maira^{6,14}, linear arrays defining concordia lower intercepts indicate the presence of inherited lead and therefore do not provide unambiguous crystallization ages. Ion-probe dating can recognize old discordant areas in zircons, but the youngest part of the concordia is linear, making mixing between young populations difficult to identify. The second method, which is that of Sm–Nd internal isochrons in garnet-bearing rocks, often fails when isotopic equilibration between clinopyroxene and garnet has not been achieved²⁰. In addition, the small spread of Sm–Nd ratios severely limits the precision of this method of dating. The closure temperature of Sm–Nd in garnet is still debated but is supposed^{20–22} to be higher than 600 °C, which should provide valuable information on the timing of eclogite exhumation.

Sguigna *et al.*²³ determined a value for the decay constant of ^{176}Lu of $(1.93 \pm 0.03) \times 10^{-11} \text{ yr}^{-1}$. An advantage of the Lu–Hf method of dating is therefore a relatively fast radiogenic ingrowth of ^{176}Hf which in particular is three times faster than that of ^{143}Nd . The uncertainty on the decay constant affects Lu–Hf ages by no more than 1.5%. The advent of plasma-source mass spectrometry, which combines a magnetic sector double-focusing mass spectrometer with a plasma source, has brought within reach for the first time precise Lu–Hf geochronology on small or Hf-poor samples. The Plasma 54 in Lyon can determine the isotopic composition of Hf on small quantities (20 ng) with an excellent external reproducibility (40 p.p.m.). Separation of Lu and Hf, blanks, standard values, and mass spectrometry are described elsewhere²⁴. Internal precision on Hf isotope compositions is usually in the range of 20–40 p.p.m. and isotope dilution produces Lu/Hf ratios currently precise to ~1%. Isolation of Sm and Nd is reduced to the separation of a rare-earth-element-enriched fraction by cation exchange chromatography (B.L. and F.A., manuscript in preparation).

High-pressure rocks are found in three internal units of the Alps known, from bottom to top, as Pennine, ophiolitic and Austro-Alpine²⁵ (Fig. 1). In the western Alps, the Dora Maira massif, the Monviso, and the Sesia-Lanzo zone each belong to one of these units (in the order as mentioned) and offer extensive outcrops of low-temperature eclogitic rocks. We have measured the Hf and Nd isotopic compositions and the Lu/Hf and Sm/Nd ratios of one eclogitic sample from each locality and in some of their constituent minerals, notably garnet, phengite and clinopyroxene. The Dora Maira coesite–pyrope–quartzite assemblage described by Chopin¹

was sampled in the classical locality of Parigi. This eclogite was equilibrated at ~700 °C and >30 kbar (ref. 1). The Monviso sample is a mafic garnet–omphacite–glaucophane eclogite equilibrated at ~450 °C and 15 kbar (ref. 26). The Sesia-Lanzo sample is a felsic eclogite equilibrated at 550 °C and >14 kbar (ref. 27). In each case, the decompression took place with no noticeable reheating.

The Lu–Hf data (Table 1) attest to the large $^{176}\text{Lu}/^{177}\text{Hf}$ ratio of garnets which reaches 3 for felsic rocks and 8 for the mafic eclogite of Monviso. Because the samples were not dissolved in pressurized bombs, the reproducibility of Lu–Hf replicate analyses of felsic

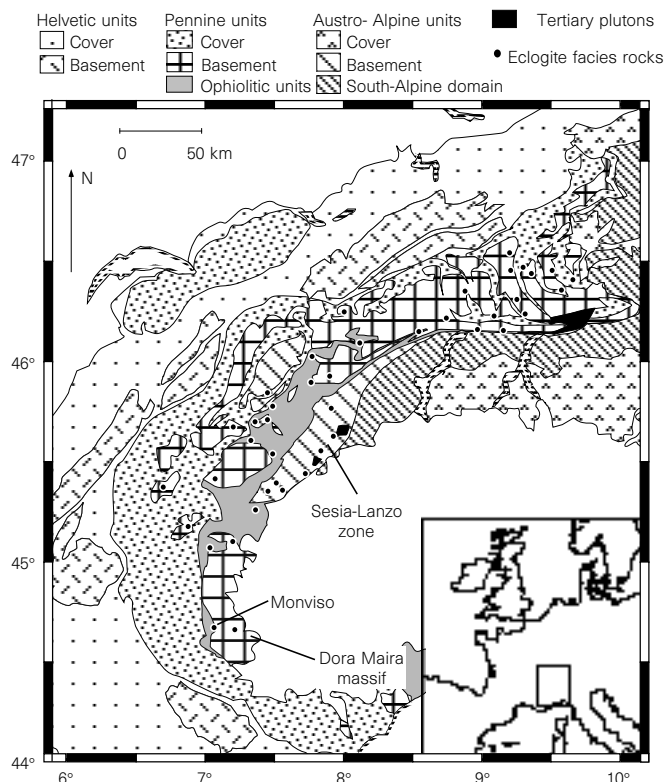


Figure 1 Sketch map of the structure of the western and central Alps with the distribution of eclogite facies rocks and sample localities indicated. The Helvetic units represent the European mainland, the Pennine units the European margin and the Tethyan oceanic crust, and the Austro-Alpine units the Apulian margin.

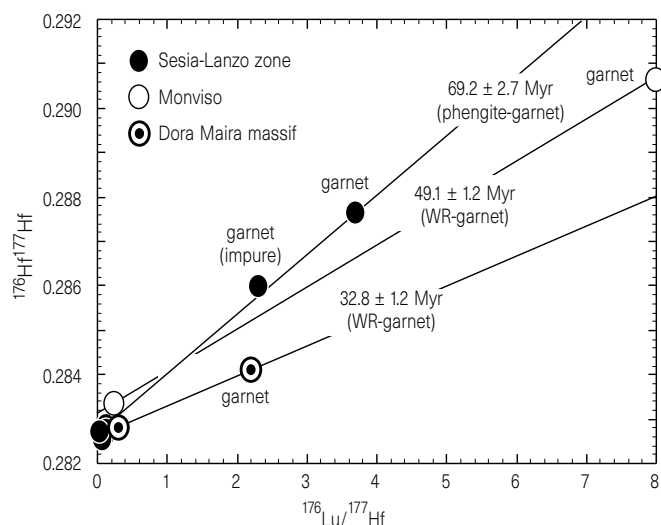


Figure 2 Lu-Hf isochrons (WR, whole rock). Phengite, clinopyroxene and whole rocks plot in the bottom left part of the diagram. Note the high value of the Lu/Hf ratio of garnets. Error bars are significantly smaller than the symbols. The rather scattered results for three replicates of a Sesia-Lanzo whole rock sample probably reflects the presence of inherited zircons. The ages, however, are well constrained by the garnet fractions.

whole rocks is not very good, suggesting that some refractory minerals may be escaping dissolution. This problem, however, should not affect the dating of mineral pairs. Because of the high Lu/Hf ratio, Hf in garnet of even very young samples is fairly radiogenic, making Lu-Hf chronology of garnet-bearing assemblages a promising technique.

The whole-rock garnet age of the Dora Maira sample is 32.8 ± 1.2 Myr (Fig. 2), which compares well with earlier 35 Myr U-Pb zircon ages¹⁵, a Sm-Nd age of 31.8 Myr on garnets from sample 15623a, and a U-Pb age of 31.4 ± 1.3 Myr on ellenbergerite^{13,14}. The whole-rock garnet age of the Monviso sample of 49.2 ± 1.2 Myr (Fig. 2) is in excellent agreement with the ^{40}Ar - ^{39}Ar ages found on phengite²⁸. The phengite-garnet age of 69.2 ± 2.7 Myr (Fig. 2) obtained on the Sesia-Lanzo sample is only marginally older than the U-Pb ages reported from zircons (65 Myr)¹¹ and sphene (66 Myr)¹². A less-purified fraction of the same garnet gives a slightly older age of 74.6 ± 4.1 Myr (Fig. 2) though still consistent within errors. The Sm-Nd data on the felsic eclogites of Dora Maira and Sesia-Lanzo are of little chronological value, most likely because of inadequate mineral purity. This shows that, owing to the high Hf content of garnets with respect to other eclogitic minerals, Lu-Hf dating is far less sensitive to the quality of mineral separation than Sm-Nd. The Monviso sample was inadvertently underspiked. The 63 ± 7 Myr Sm-Nd age obtained from the clinopyroxene-phengite pair from the Sesia-Lanzo sample and the crude 40 ± 10 Myr clinopyroxene-garnet Sm-Nd age acquired through a calibration of the Sm/Nd beam ratio using standard solutions are, however, consistent with the Lu-Hf data.

The significance of ages depends on whether the temperature at which exchanges of parent and daughter isotopes are frozen, the closure temperature T_c (ref. 29), is higher or lower than the temperature of crystallization. U-Pb ages of concordant zircons date the crystallization of this mineral. By contrast, K-Ar ages generally date closure at low temperature. Because little is known about diffusion of Hf in common minerals, it is not clear whether Lu-Hf ages of eclogitic garnets reflect a closure temperature or the time of crystallization. Hafnium in granitic liquids diffuses more slowly than neodymium³⁰. The ionic radius³¹ of six-fold-coordinated Hf^{4+} (0.083 nm) in crystals is intermediate between that of Mg^{2+} (0.072 nm) and that of the trivalent rare-earth elements (0.11 nm). Comparison with the closure temperature of Fe-Mg thermometers³² and Sm-Nd chronometers²⁰⁻²² suggests that the closure temperature of the Lu-Hf system in garnet-bearing assemblages is most probably in excess of 600 °C. This chronometer therefore dates the early stage of exhumation in Dora Maira, and the

high-pressure metamorphic event itself in the low-temperature eclogites of Monviso and Sesia-Lanzo.

The exhumation of eclogites seems to be very rapid. At Dora Maira, the U-Pb crystallization age of zircons, the high-temperature Sm-Nd and Lu-Hf closure ages, and the K-Ar age of the retrogressive phengites crystallized in the greenschist facies, all coincide within error (~ 3 Myr). The coesite-pyroxene quartzites therefore seem to have been decompressed from 30 to 3 kbar at a rate of the order of 3 centimetres per year, well outside the range accepted for erosion rates. Similar arguments also support fast exhumation for the Sesia-Lanzo zone and Monviso.

The age of the early stage of exhumation obtained by the Lu-Hf method on the same type of mineral is older when the structural position of the units that host the eclogites is higher. The Lu-Hf ages at Monviso and Sesia-Lanzo clearly predate the Late Eocene-Early Oligocene collision between the European and Apulian plates, which is dated as the time when material from either continental margin engaged into the Pennine and Austro-Alpine nappes^{25,33,34}. The youngest record of eclogite exhumation actually coincides within errors with the collision and is found in the Pennine unit at Dora Maira and Alpe Arami in the central Alps¹⁸. It predates by less than a few Myr the intense 31-Myr-old magmatic activity represented by the emplacement of the granitic pluton at Bergell³⁵ and other localities³⁶, and by the eruption of the widespread andesites found as clasts in the Tavayannaz and Champsaur formations³⁷. The suggestion that at 30 kbar eclogite-facies rocks could partially melt³⁸ is supported by the contemporaneity of their exhumation with magmatism and by the similarity of Nd isotopic compositions in the felsic eclogites and in Alpine granites³⁹.

The geochronological evidence indicates that the process of burial and exhumation of crustal rocks is rapid with respect to the overall duration of the high-pressure conditions. Some eclogites are already brought to the surface well before the protolith of others even become buried. The exhumation of the Sesia-Lanzo zone was complete ~ 35 Myr before the Dora Maira massif was metamorphosed in the eclogite facies. Some eclogites therefore rise through a near steady-state mechanism apparently unrelated to the collision, possibly through prism flow⁴⁰ in a subduction environment. □

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A sexually selected character displacement in flycatchers reinforces premating isolation

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Theory suggests that natural selection against the production of unfit hybrids may reinforce barriers to gene flow, eventually leading to reproductive isolation of differentiated populations^{1–4}. This mode of speciation may be achieved by female choice selecting for a divergence in male secondary sexual traits that facilitates species recognition. Although intuitively appealing, conclusive evidence for such reinforcement is generally lacking^{5–8}, and serious doubts have been raised about its validity^{9–11}. We have tested key predictions of the reinforcement hypothesis on the European, black-and-white, *Ficedula* flycatchers, using molecular techniques, field observations and mate choice experiments. In populations where two species coexist, we show that female choice selects for a divergence in male plumage colour and that the resulting character displacement reduces the frequency of hybridization.

The current geographical distribution of European *Ficedula* flycatcher forms has led researchers to suggest that a sympatric divergence in secondary sexual characters has occurred^{12–14}. In a large area in Central and Eastern Europe the breeding distribution overlaps for two clearly differentiated flycatchers, the brown form of

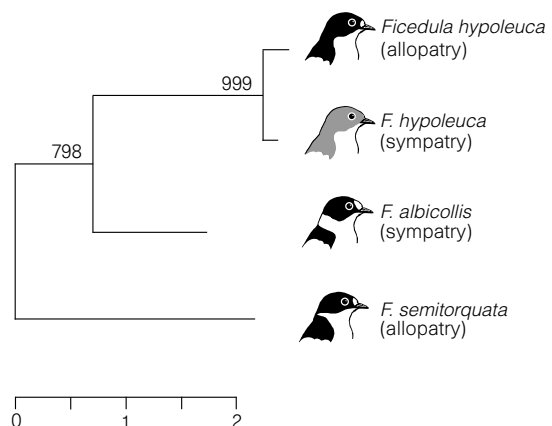


Figure 1 Phylogenetic relationships among sympatric and allopatric flycatchers based on mitochondrial DNA sequences. A neighbour-joining tree is presented with genetic distances drawn to scale. The scale refers to Kimura 2-parameter distances (%). Values at the nodes represent bootstrap replication scores based on 1,000 resamplings. The phylogenetic reconstruction suggests that the plumage characters in sympatry are derived traits, supporting the argument of a sympatric character divergence.

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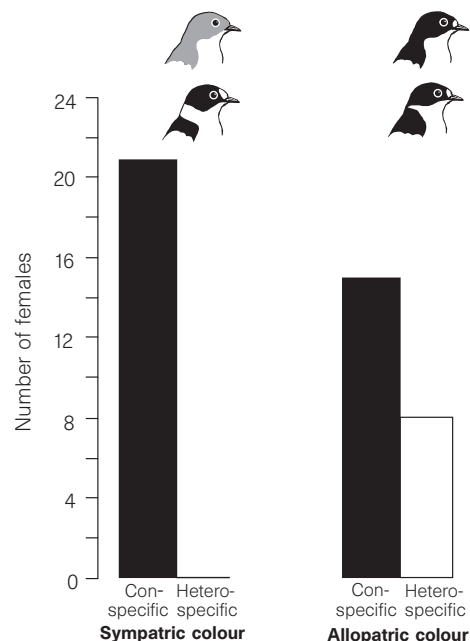


Figure 2 Species recognition by female pied and collared flycatchers from sympatric populations. Species assortative mating was significantly reduced when males of the two species had plumage characteristics typical of allopatric populations (small difference) compared to when the males had plumage characteristics typical of sympatric populations (large difference: $\chi^2 = 6.7$, d.f. = 1, $P = 0.009$). In the case of allopatric colours, 5 out of 12 female pied flycatchers and 3 out of 11 female collared flycatchers chose the heterospecific male.

the pied flycatcher (*Ficedula hypoleuca*) and the black-and-white collared flycatcher (*Ficedula albicollis*). These two forms are restricted to the zone of overlap and adjoining areas^{12,13}. The distributions of allopatric flycatchers encircle the area of sympatry¹². Different allopatric flycatchers resemble each other and are intermediate to the sympatric ones in plumage characteristics. The majority of males are black-and-white but with smaller patches of white on the forehead, neck and primaries than those of a typical collared flycatcher. Hypotheses on evolutionary history based on geographical distributions have been problematic because the phylogenetic relations between the various allopatric and sympatric forms have remained unresolved. Accordingly, it has been difficult to assess whether the colours of the sympatric forms represent the derived character states¹². Using mitochondrial DNA analysis, we found that one of the phenotypically intermediate allopatric forms, the semicollared flycatcher (*Ficedula semitorquata*, here given the status of species), is the most genetically divergent form (Fig. 1). Allopatric pied flycatchers resemble semicollared flycatchers phenotypically, but are only distantly related to that species (Fig. 1). These results indicate that the coloration occurring in allopatry might represent the ancestral state, whereas the colorations occurring in sympatry are derived traits. Hence, the phylogenetic relations suggest that a sympatric divergence in plumage characteristics has occurred.

Reinforcement can be considered an evolutionary response to maladaptive mating behaviour. Selection may favour assortative mating when hybridization is detrimental to fitness¹⁵. Breeding records and fitness data were collected from sympatric populations in the Czech Republic and Slovakia¹⁶. According to the reinforcement hypothesis we expected to find negative fitness consequences from hybridization and a bias towards species-assortative mating, reflecting a history of selection against hybridization. A total of 2,348 mating events occurring in temporal and spatial sympatry

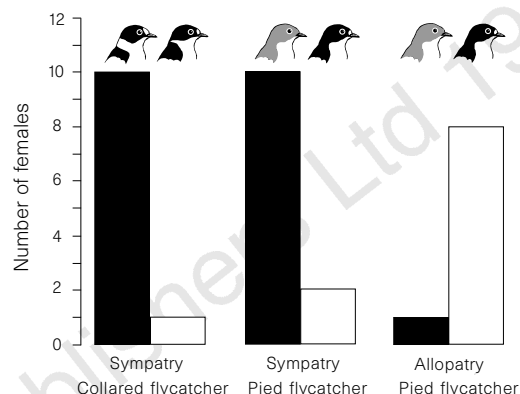


Figure 3 Mate preferences of female flycatchers. Left, female collared flycatchers preferred male conspecifics with large patches of white to those with reduced patch sizes ($\chi^2 = 7.4$, d.f. = 1, $P = 0.007$). Centre, sympatric female pied flycatchers preferred brown coloured male conspecifics to black-and-white ones ($\chi^2 = 5.3$, d.f. = 1, $P = 0.027$). Right, allopatric female pied flycatchers (previously published data²⁶) preferred black-and-white coloured male conspecifics to brown ones ($\chi^2 = 5.4$, d.f. = 1, $P = 0.020$). Mate preferences of sympatric female pied flycatchers were opposite to those of allopatric ones ($\chi^2 = 8.1$, d.f. = 1, $P = 0.005$).

were analysed. Of these, 2,226 (94.8%) were species assortative, 61 (2.6%) were heterospecific and 61 (2.6%) involved one hybrid¹⁶. If mating had been random we would have expected a frequency of 13.8% heterospecific matings based on temporal and spatial availability of potential mates. The observed frequency deviates significantly from the expected one ($\chi^2 = 235.3$, d.f. = 1, $P < 0.0001$). Hence, mating is strongly skewed towards species assortative ones. Hybrids had very low fertility. In pure and mixed-species pairs ($n = 332$) only 4.9% of the eggs failed to hatch, compared to 74.1% in pairs with a hybrid ($n = 60$; $t = -25.1$, d.f. = 391, $P < 0.0001$). Hence, there is a substantial selection potential against hybridization.

Male plumage characteristics that resemble those of allopatric flycatchers are found at low frequencies among sympatric pied and collared flycatchers¹²⁻¹⁴. Therefore, the degree of interspecific divergence in plumage colour varies. The existence of such variation makes it possible to investigate whether selection for sympatric character divergence is currently operating.

According to the reinforcement hypothesis the ultimate explanation for a sympatric character divergence is that it reduces the probability of hybridization. Two experiments were designed to investigate whether divergence in plumage colour facilitates species recognition. In aviary trials, females of both species from sympatric populations could choose between one male of each species. First, females of both species were allowed to choose between males with divergent plumage characteristics, typical of sympatric populations. All females responding by building a nest chose the conspecific male (Fig. 2). Second, the females were allowed to choose between males with more similar colours, typical of allopatric flycatchers. A high proportion of the females then built their nest in the nest-box advertised by the heterospecific male (Fig. 2). Hence, divergence in plumage characteristics helps species recognition.

In most models of the reinforcement hypothesis it has been

assumed that an eventual development of assortative mating is caused by the action of natural selection on an assortative mating locus^{10,17–19}. However, sexual selection in the form of female preferences for male traits may enhance the reinforcement process^{4,20}. When hybridization is detrimental to fitness, female choice for divergent male characteristics in sympatry would parallel the 'choice of good genes process' of sexual selection^{21–23}. We tested whether the observed character divergence of the flycatchers was related to a female preference for divergent male characteristics. In one experiment, female collared flycatchers could choose between a male conspecific with large patches of white (forehead, neck and primaries) and a conspecific with reduced white patches, resembling an allopatric flycatcher. The females preferred the one with the larger white patches (Fig. 3). In another experiment, female pied flycatchers from the same mixed populations could choose between one dull, brown and one black-and-white conspecific male. The females preferred the dull, brown male (Fig. 3). Hence, in sympatry, females of the two species show preference for divergent male characteristics.

In allopatry, a black-and-white colour of a male pied flycatcher indicates a high viability of the carrier^{24,25} and females prefer such males to dull, female-like brown ones²⁶ (Fig. 3). In sympatry with collared flycatchers, mate preferences of female pied flycatchers are opposite to those in allopatry (Fig. 3). To our knowledge, the present study is the first on mate choice in any species to demonstrate a female preference for the least conspicuous male traits²⁷. By choosing dull, brown males, female pied flycatchers increase the probability of obtaining a conspecific mate. Hence, in the case of this species it appears that selection against hybridization in sympatry with the collared flycatcher overrides the general trend of sexual selection for elaborate traits.

In conclusion, female choice selects for a character displacement of male plumage traits in sympatric flycatchers that helps species recognition. Character displacement and assortative mating are adaptive, because heterospecific matings result in hybrid offspring with very low fertility. As would be expected from a history of selection against hybridization there was a strong bias towards species-assortative mating in sympatric populations. Hence, there is evidence for ongoing speciation by reinforcement of premating isolation in sympatric pied and collared flycatchers enhanced by a sexually selected character divergence in male plumage colour. □

Methods

Sequencing and phylogenetic analysis. Genomic DNA was isolated from blood samples of sympatric Czech pied (*F. hypoleuca*; $n = 8$) and collared flycatchers (*F. albicollis*; $n = 10$), and from allopatric Spanish pied flycatchers ($n = 10$). DNA from the semicollared flycatcher (*F. semitorquata*) was isolated from a museum specimen of Armenian origin. We used bluethroat (*Luscinia svecica*) for outgroup-rooting. The mitochondrial NDH dehydrogenase subunit 6 gene (ND6) was enzymatically amplified using conserved primers in the control region (5'-GGTCTCTGAGAAACACGAT-3') and the tRNA^{Pro} gene (5'-TCCATCTCCAGCTCCCAAAGC-3'). Several sequencing primers were used (data available on request). The complete ND6 sequence (519 base pairs) was collected in all specimens using the Amplitaq Cycle sequencing kit (Perkin Elmer) and Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham). The average genetic distance from the outgroup, *L. svecica*, to the flycatcher species was 18.2%. Intraspecific variation was low, with a maximum of two steps in all pairwise comparisons. With reference to the most common genotype in each species, substitutions were present at six nucleotide positions that were consequently omitted from the phylogenetic analysis. Kimura 2-parameter distances with a transition/transversion ratio set at 20 and the neighbour-joining method were used for phylogeny reconstruction²⁸. Sequences have been deposited in the EMBL database (accession numbers Y10215; Y10308–Y10311).

Hybrid fitness and mating pattern. Data on hatching success and frequency of hybridization were collected from 14 mixed-species populations in the Czech Republic and Slovakia¹⁶. Classification of hybrids versus pure individuals is

based on several species-specific morphological traits and vocalizations that are intermediate in hybrids^{12,16}. Estimates of expected and observed frequencies of hybridization are based on years in which individuals of both species were found breeding within the same locality (temporal and spatial sympatry).

Mate choice and species recognition. Experiments in four outdoor aviaries ($3 \times 3 \times 1.9$ m)²⁹ were conducted during spring 1995 and 1996 at Dlouhá Loučka, Czech Republic. The aviaries contained one female compartment and two male compartments. The males advertised separate nest-boxes. The females could see both males but the males could not see each other, and nylon netting prevented the birds from entering other compartments. Food and water were freely available and dried grass and leaves served as nesting material for females. Birds were captured from different nest-box areas 0–30 km away from the aviaries, and birds within each trial were captured at different sites to avoid previous experience. Males were habituated to aviaries for 24 h whereafter females were introduced. The chosen male was regarded as the one advertising the nest-box in which the female built her nest. To avoid possible correlates of plumage colour we used colour manipulations²⁶ to produce differences. Previous studies show that pied and collared flycatchers respond similarly to colour manipulated and naturally coloured birds^{14,26}. In the species recognition experiments (Fig. 2) each pair of males ($n = 30$ pairs) was used to test the response of one female of each species. In the case of sympatric colours, 11 female collared and 10 female pied flycatchers responded by building a nest, and in the case of allopatric colours, 11 female collared and 12 female pied flycatchers responded. We used a balanced design with respect to relative position of the males in the aviaries and order of presentation. In the mate choice experiments of sympatric populations (Fig. 3, left and centre) each pair of males (collared flycatcher, $n = 15$ pairs; pied flycatcher: $n = 13$ pairs) was used to test the response of one conspecific female. Pairs of males were selected on basis of similarity in age, size and initial plumage colour (using birds with typical sympatric colours only). One randomly chosen male within each pair was colour-manipulated using Nyanzol²⁶ to resemble black-and-white, allopatric male flycatchers. We used a balanced design with respect to relative position of the painted and the unpainted males in the aviaries. Similar methods were used for mate choice of allopatric pied flycatchers (Fig. 3, right)²⁶. The birds used in experiments were captured at different localities, though from a geographically restricted area (30 km radius). Field data (mating order of colour types) from other flycatcher populations^{12,30} are consistent with the experimental results obtained, suggesting that the observed differences in mate choice apply to allopatric and sympatric populations in general. Nevertheless, note that these characteristics may vary among localities and future efforts should be made to conduct similar tests in sympatric and allopatric populations throughout the species range.

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Dissociating types of mental computation

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A fundamental issue in the study of cognition and the brain is the nature of mental computation. How far does this depend on internally represented systems of rules, expressed as strings of symbols with a syntax, as opposed to more distributed neural systems, operating subsymbolically and without syntax? The mental representation of the regular and irregular past tense of the English verb has become a crucial test case for this debate. Single-mechanism approaches argue that current multilayer connectionist networks can account for the learning and representation both of regular and of irregular forms^{1,2}. Dual-mechanism approaches, although accepting connectionist accounts for the irregular forms, argue that a symbolic, rule-based system is required to explain the properties of the regular past tense and, by extension, the properties of language and cognition in general^{3–5}. We show here that the regular and irregular past tense are supported by different neural systems, which can become dissociated by damage to the brain^{6,7}. This is evidence for functional and neurological distinctions in the types of mental computation that support these different aspects of linguistic and cognitive performance.

This research brings new evidence to bear on the mental representation of the regular and irregular past tense in English, by examining the performance of aphasic patients with acquired neurological damage. If regular and irregular forms are mentally represented and processed in fundamentally the same way, then both should be affected in similar ways by damage to the brain that disrupts morphological processing systems. If there are two separate underlying systems engaged, respectively, by the regular and irregular morphology, then it should be possible to find dissociations in performance between these two morphological domains.

We selected two aphasic patients (J.G. and D.E.) with well documented difficulties in the comprehension and production of inflected forms in English⁸. Both patients have typical 'agrammatic' speech, which is hesitant and rarely contains inflected words^{8–10}. In tests of their ability to interpret morphologically complex words, they can access the stems of such words, but have consistent difficulties in interpreting the combination of the stem with an

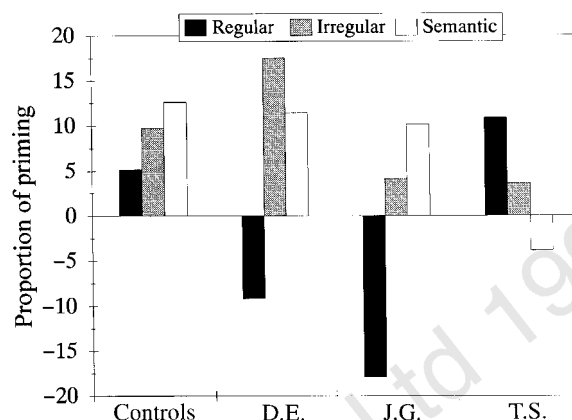


Figure 1 Priming effects across patients (D.E., J.G., T.S.) and control subjects for regular and irregular past tense primes and for semantic primes. Effects are expressed as response proportions (mean priming effect for each condition as a proportion of mean control reaction-time for that condition) to normalize for differences in base reaction-time between subjects.

inflectional affix: as in forms such as 'jumps' or 'smiling' (decomposable into {jump} + {-s} and {smile} + {-ing})^{8,9}. Such patients should also have problems in the access and interpretation of regular past tense forms such as 'jumped' or 'smiled' which again involve the combination of a stem with a regular inflectional affix. The crucial question is whether they will show the same deficit for irregular forms such as 'gave' or 'taught', where the morphological relationship between prime and target does not involve the same types of combinatorial operation.

To test this required appropriate experimental tasks, using auditory rather than visual materials, and avoiding spoken responses, because both patients have difficulty in reading text and in producing speech^{11,12}. Instead, we used an auditory priming task, where a spoken target word is immediately preceded by a spoken prime word, and the listener makes a timed lexical decision response to the target ('is this a word or not?'). For unimpaired subjects, responses to a target word speed up when it is preceded by a morphologically related prime (as in the prime/target sequence 'jumped/jump' or by a semantically related prime (as in 'swan/goose'), but not when the relationship is purely phonological (as in 'grave/grave'). Earlier studies confirmed that these patients could perform this task and that they showed normal levels of semantic priming^{9,10,13}.

The two patients and six age-matched normal controls were tested in an auditory priming experiment that contrasted priming effects for regular and irregular past tenses (Table 1). For the regular past tense condition, the target word (for example 'jump') shares its stem morpheme with the prime, always a regular inflected past tense form (for example 'jumped'), and would normally show priming relative to a control prime (for example 'locked') that has no morphological or semantic relation with the target. Similarly, for the irregular past tense conditions, the target (such as 'give') shares an underlying morpheme with the prime, always an irregular inflected form (such as 'gave'), and this should also produce priming relative to an unrelated prime (such as 'shows'). In each case, we assume that priming reflects the repeated activation, by the prime and the target, of the same underlying lexical representations.

For comparison, we included a semantic priming condition (Table 1), where the prime word (such as 'swan') is preceded by a semantically (but not associatively) related prime word ('goose'), which should elicit priming for both controls and patients, and a phonological condition where the prime and target were only phonologically related (as in pairs such as 'grave/grave').

Control subjects, consistent with results for other groups of

Table 1 Sample stimulus set

Condition	Test prime	Control prime	Target word
Regular past	jumped	locked	jump
Irregular past	found	shows	find
Semantic	swan	hay	goose
Phonological	gravy	sherry	grave

Table 2 Mean lexical decision response times and error rate

Condition	Controls		D.E.		J.G.		T.S.	
	Test	Control	Test	Control	Test	Control	Test	Control
Regular past	809 (0)	852 (2)	1,115 (10)	1,021 (15)	1,150 (15)	976 (15)	998 (0)	1,119 (0)
Irregular past	805 (0)	892 (2)	1,047 (10)	1,269 (10)	1,041 (10)	1,086 (15)	1,056 (0)	1,106 (0)
Semantic	707 (2)	809 (5)	772 (0)	871 (5)	848 (5)	943 (15)	1,250 (0)	1,203 (0)
Phonological	867 (0)	897 (0)	950 (10)	1,156 (25)	1,095 (10)	1,146 (25)	1,085 (0)	1,126 (0)

Times are in ms, error rate is in percent (in parentheses).

unimpaired adults in auditory priming tasks, showed faster responses both for regular and irregular past tense forms (Table 2), with no interaction between regularity and priming ($\text{MinF}'[1, 39] = 1.56, P > 0.20$). This pattern held for each control subject individually, none of whom showed an interaction between regularity and priming. It is also the pattern shown by a group of 25 young adults tested on the same materials. J.G. and D.E., in contrast, produced distinctively different responses to the two types of past tense prime. They showed positive priming effects for the irregular past tense, but significant interference for the regular past tense. This was reflected in a significant interaction between regularity and priming for both J.G. ($F[1, 27] = 6.33, P < 0.01$) and D.E. ($F[1, 27] = 4.68, P < 0.05$). Consistent with our earlier work with these patients, they show impairments for the regular inflectional morphology, but this does not extend to the irregular morphology as well.

This cannot be interpreted as a phonological priming effect, based on the phonetic similarity between prime and target word, because the regular past tense primes, with their unchanged stems (as in 'jumped/jump' or 'danced/dance'), are phonologically more similar to their targets than the irregular past tense primes, which typically undergo some form of stem change (as in 'gave/give', 'crept/creep'). Nonetheless, it is the less similar irregular past tenses that prime, and not the regulars. Furthermore, purely phonological primes did not produce significant effects, either for the controls ($\text{MinF}'[1, 26] = 1.22$, not significant), or for J.G. ($F < 1$) or D.E. ($F[1, 13] = 3.23, P = 0.96$).

Turning to the semantic primes, these produced consistent effects throughout, with significant facilitation both for the controls ($\text{MinF}'[1, 22] = 16.99, P < 0.001$), and for J.G. ($F[1, 13] = 10.18, P < 0.001$) and D.E. ($F[1, 15] = 4.32, P < 0.05$). This suggests a closer relationship, across subject groups, between semantic primes and irregular past tense primes than between semantic and regular primes.

This relationship was sustained in subsequent testing of a third patient (T.S.), also classified as 'agrammatic', but with right as well as left hemisphere damage. T.S. produces the opposite pattern to D.E. and J.G., with normal performance for the regular past tense ($F[1, 18] = 4.21, P = 0.05$) and no priming for the irregular past tense ($F < 1$), accompanied by no phonological or semantic priming ($F < 1$). This double dissociation for the regular and irregular morphology is plotted in Fig. 1, together with the pattern of semantic priming effects. Statistical confirmation for this dissociation comes from two additional analyses of variance, where patient

is entered as a factor. In each analysis there is a three-way interaction between patient, regularity and priming: for the comparison between D.E. and T.S., $F[1, 26] = 4.63, P = 0.041$; for J.G. and T.S., $F[1, 27] = 4.99, P = 0.034$.

These results make two important points about types of mental computation and their neural substrate. First, they demonstrate that the regular and irregular past tenses are neurologically dissociable, with the appropriate on-line tasks revealing deficits in the access of regular but not irregular inflected forms for D.E. and J.G., and the converse effect for T.S. Second, they suggest that the two morphological categories ally themselves with different types of mental computation. The deficits of D.E. and J.G. for the regular past tense are consistent with earlier evidence that these patients have marked deficits in combinatorial operations involving morphologically complex words. The irregular past tenses, which have been relatively spared for D.E. and J.G., seem to fit with the pattern of evidence that suggests these patients can still access the semantic properties of morphologically simple words. T.S., in contrast, who shows no semantic priming, also shows no priming for the irregular past tense relative to the normal levels of priming he achieves for the regular past tense.

The specific challenge this poses for single-mechanism connectionist accounts is to show how a neural network, exposed to the same training input as a child learning English, can learn to partition its representations of regular and irregular morphology in such a way that (1) its end state is functionally separable into apparently combinatorial and non-combinatorial operations, and (2) these two aspects of its function are doubly dissociable by different types of damage to the network. □

Methods

Subjects. The 6 control subjects ranged in age from 58 to 66 years and had no history of neurological impairment.

D.E. (aged 41 years at testing) is a right-handed man who suffered a cardiovascular accident (CVA) in 1970. A magnetic resonance imaging (MRI) scan taken in 1996 revealed a large left-hemisphere (LH) lesion affecting the middle and posterior parts of the frontal lobe and most of the temporal lobe. There was no damage to the parietal lobe and no abnormality in the right hemisphere (RH).

J.G. (aged 66 at testing) is a right-handed man who suffered a left temporo-parietal CVA in 1980. A positron-emission tomography (PET) scan carried out in 1992 showed no active metabolism in his LH.

T.S. (aged 50 at testing) is a right-handed man with a 16-year history of vascular problems culminating in a RH middle cerebral haemorrhage in 1995. A computerized tomography (CT) scan in October 1996 revealed RH inferior parietal and temporal damage and changes to frontal and occipital lobes, as well as patchy ischaemic damage to LH frontal, parietal and temporal lobes.

We assessed T.S.'s language comprehension and production using standard tests. On the BDAE¹⁴ he showed the profile typical of a Broca's aphasic with a mild comprehension deficit. His speech output was non-fluent with minimal syntax and few complex words. Describing the 'Cookie theft' picture¹⁴ he said: kitchen room.. lady washing.. accident sink flow.. water.. splash, splash, splash.. um.. play dry.. boy stool.. cupboard.. cookie jar.. friends.. hands". In additional tests he could repeat single words (56/60 correct) but not sentences (0/36 correct)¹⁵. His many errors (16/34) on a 3-picture sentence-picture matching task were all reverse role distractors¹⁶. He was poor at making grammatical judgements, scoring 46/60 correct¹⁷. This is an overall pattern of performance typical of 'agrammatic' aphasics.

Materials and design. Twenty pairs of each prime-target type (Table 1), with targets matched for frequency over conditions, and combined with real word and nonword filler pairs, were assigned to two experimental versions. These were presented to patients and controls in two testing sessions 4 weeks apart, such that each target word was heard only once in each session, preceded by either the test or control prime. There was a 250 ms interval between prime and target, and a 3 s interval between trials. Lexical decision responses were made by pressing response keys labelled either yes or no.

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Distortions of visuotopic map match orientation singularities in primary visual cortex

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The map of orientation columns in primary visual cortex (V1) is known to show strong local distortions, with a generally smooth progression of orientation preference across extended regions of cortex, interrupted by sharp jumps (fractures) and point singularities^{1–4}. The map of visual space on V1, in contrast, has been assumed to be locally smooth and isotropic. We find, on the contrary, that the map of visual space on cat V1 shows strong and systematic local distortions in register with inhomogeneities in the orientation map, with the rate of receptive field movement across cortex being largely proportional to the local rate of change of orientation. This suggests possible systematic local variations in the functional connectivity of short-range lateral connections that underlie local cortical processing.

A detailed knowledge of the map of visuotopy on V1 is critical to understanding the cortical representation and processing of visual input. Early electrophysiological recordings of visual field position in V1 suggesting that the map of visual space is smooth and locally isotropic^{5–7}. A systematic change in cortical magnification on moving from the centre to the periphery of visual space was believed to be the only inhomogeneity present in this map; any deviations from smoothness were attributed to random scatter in the positions of receptive fields (RFs). The advent of optical imaging has made it possible to examine whether the apparent scatter in RF positions was random or showed a systematic relation to the distribution of orientation columns.

We have used a combination of optical imaging of intrinsic signals (that is, changes in cortical reflectance linked to metabolic changes resulting from local neural activity^{8,9}) and extracellular recording of RFs to explore the relation between maps of space and

orientation in cat V1. Optical images of orientation columns (Fig. 1a) show a characteristic pattern, with fields of constant or slowly changing orientation preference interrupted by local discontinuities ('fractures' or 'singularities')^{2–4}. Multi-unit RFs recorded near fractures show a distinctly non-uniform movement through space, with rapid jumps across fractures and high overlap in regions of uniform orientation (Fig. 1a–c). A longer sequence of roughly uniformly spaced recording sites traversing a singularity shows the same pattern, of RFs clustered within an orientation zone and no overlap between zones (Fig. 1d). This gives the corresponding region of visual space the appearance of being tiled by abutting clusters of RFs, as opposed to being covered by a smooth progression of RFs. Note that at site 2, in the fracture, we recorded distinct units that responded at two orthogonal orientations at the same electrode position. Their receptive fields (marked 2 and 2') overlapped with RFs of corresponding orientation preference obtained at other recording positions. This extends the idea that some orientation singularities are infinitesimal in extent, with neurons of one orientation preference adjacent to neurons of orthogonal orientation preferences^{1,4}. Recordings across cortical regions of smoothly changing orientation show that the saltatory RF movement illustrated in Fig. 1a–d defines the extremes of a more general linear relation between rates of RF movement and orientation change (Fig. 1e). At the same time, vertical penetrations through the upper cortical layers at any recording site produce multi-unit RFs that are largely superimposed on each other, with little scatter in either size or position (Fig. 1f). This suggests that inhomogeneities in retinotopy are not diluted significantly by RF scatter and that the relation between retinotopy and the orientation map is maintained faithfully down cortical columns.

We quantified the relation between RF movement and orientation shift by measuring changes in RF position as a function of changes in orientation preference between pairs of neighbouring recording sites (Fig. 2a; also see Methods). The values thus obtained were then divided by the corresponding intersite distance on the cortex to give the rate of RF movement across the cortex as a function of the rate of change of orientation (Fig. 2b). Two features of these pooled data (taken from 143 electrode penetrations, in 20 series of recording sites in 13 animals) are noteworthy. We see that the local rate of RF movement is proportional to the local rate of change of orientation (Fig. 2b; Table 1). This would be expected to lead to strong and systematic modulations in the overall visuotopic map in register with the varying rates of change of orientation over the cortical surface (Fig. 3). With RF movement normalized by RF dimensions (see Methods and Fig. 2 legend) the linear relation between rates of RF movement and orientation change holds out to all eccentricities of V1 tested, scaling appropriately with RF size and cortical magnification⁷. The next noteworthy feature is the slope of the regression line in Fig. 2a relating shifts of RF centre to changes in orientation between neighbouring recording sites. The value of 0.0106 RF diameters per degree of change in orientation (Table 1) translates to a shift of 1.9 RF diameters over a 180° cycle of orientation preferences. This means that if we were to map the RF from a cortical site of any particular orientation preference and then move one cycle of orientation in any arbitrary direction on cortex to another site of the same orientation preference, we would have moved about 2 RF diameters, that is, to a non-overlapping region of visual space.

A number of precautions were taken to minimize, and then check for, bias in our data and to check the robustness of our results. Electrode recordings were made 'blind' so as not to be influenced by knowledge of the orientation map. We checked that there were no systematic errors of measurement (Fig. 2c legend) by confirming the lack of correlations in scatter plots of RF shift versus intersite distance ($R^2 = 0.06$ (see supplementary information); Fig. 2c) or of orientation shift versus intersite distance ($R^2 = 0.09$ (see supplementary information)) in our pooled data. Moreover, these

Table 1 Slopes and correlation coefficients for experimental results compared with values from Monte Carlo simulations

	Rate of RF movement (fractions of RF diameters per 100 μm of cortex) versus rate of change of orientation (degrees per 100 μm of cortex)			Shift in RF (fractions of RF diameters) versus change in orientation (degrees)		
	Slope ($\pm$ s.e.m.)	R^2	N	Slope ($\pm$ s.e.m.)	R^2	N
Real data	0.0110 ($\pm$ 0.00066)	0.66	143	0.0106 ($\pm$ 0.00057)	0.71	143
Monte Carlo simulation 1 (linear map + scatter)	0.002 ($\pm$ 0.00034)	0.04	838	-0.0004 ($\pm$ 0.00032)	0.002	838
Monte Carlo simulation 2 (real data + scatter)	0.0119 ($\pm$ 0.0002)	0.79	1000	0.0105 ($\pm$ 0.00019)	0.75	1000
Data from Fig. 4c				0.0140 ($\pm$ 0.0008)	0.95	15

Row 1, results from Fig. 2a,b, row 2, Monte Carlo simulation of null hypothesis that a linear map plus scatter can account for the experimental results; row 3, simulation of the effect of measurement uncertainty on results; row 4, results from Fig. 4 (not part of pooled data).

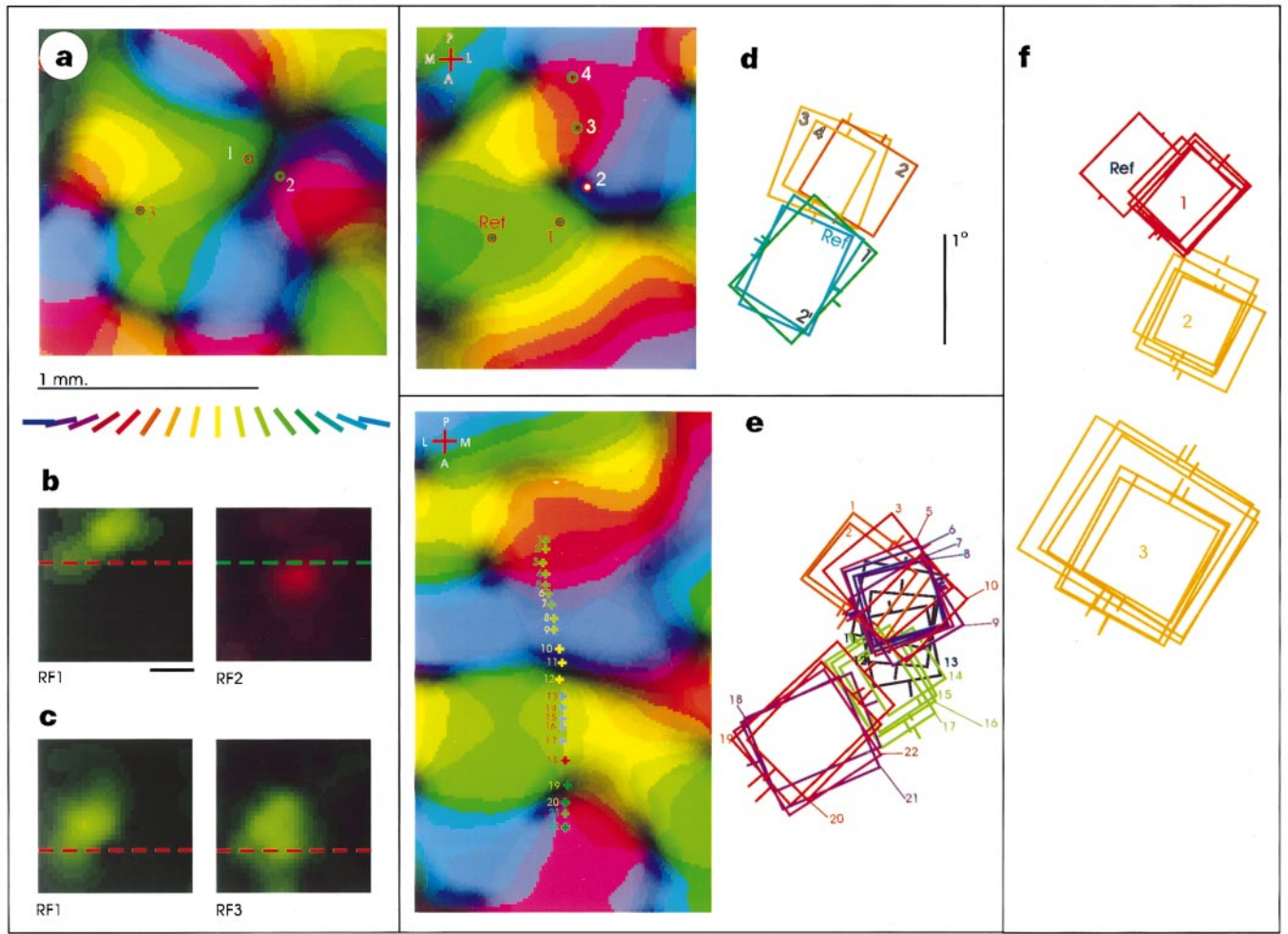


Figure 1 Rate of movement of RFs is proportional to the rate of change of orientation over the cortical surface. RFs recorded at different depths, down to 500 μm , from any cortical site show little scatter in size or centre position. **a**, Optical map of orientation columns in V1 with three electrode recording sites. Orientation preference is shown binned into 11.25° intervals and colour coded starting with blue (bin 0° to 11.25°; see key below **a**; this code is used for all optical images and RF profiles). The brightness of each colour indicates the strength of the computed orientation vector, with dark points or lines indicating singularities or fractures. Scale bar, 1 mm (same for all optical images in this figure). **b**, Grid maps of RFs measured simultaneously from sites 1 and 2 in **a**, separated by only 130 μm but across a fracture, show little overlap. Scale bar, 1°; a broken line is drawn through corresponding portions of each RF map in **b** and **c**, for ease of comparison. **c**, Grid maps of RFs from sites 1 and 3, 400 μm apart but in a region of uniform orientation, show significant overlap. **d**, Optical image (left) from a different experiment, with numbered recording sites corresponding to the RFs on the right. 'Ref' marks fixed reference RF. See text for details. Scale bar, 1° (same

for RF maps in **d-f**). **e**, Left, optical image and recording sites in a region of V1 with relatively smoothly varying orientation (red, (45°) to blue (0°), posterior half). Right, RFs corresponding to the recording sites 1 to 11 move smoothly through space before clustering on top of each other just posterior to fracture at site 11. Sites in green (~135°) and red (~45°) give non-overlapping clusters of RFs. Note that we have consistently used the hand-mapped values of orientation preference for all quantitative analyses on all RF data. Away from singularities, hand-mapped values agreed closely with optically imaged orientation preferences, although near singularities they were occasionally inconsistent. **f**, Scatter in RF position and size. Three examples, at three different eccentricities, of RF profiles obtained at 50 μm intervals in vertical penetrations down to 500 μm (shown with respect to their common reference RF, marked 'Ref'). For clarity, RFs that were indistinguishable from each other have not been marked separately. Over four such experiments (24 sites), RFs showed an r.m.s. scatter in position of $0.1 \times$ mean RF size, and an r.m.s. variation in RF size of $0.1 \times$ mean RF size.

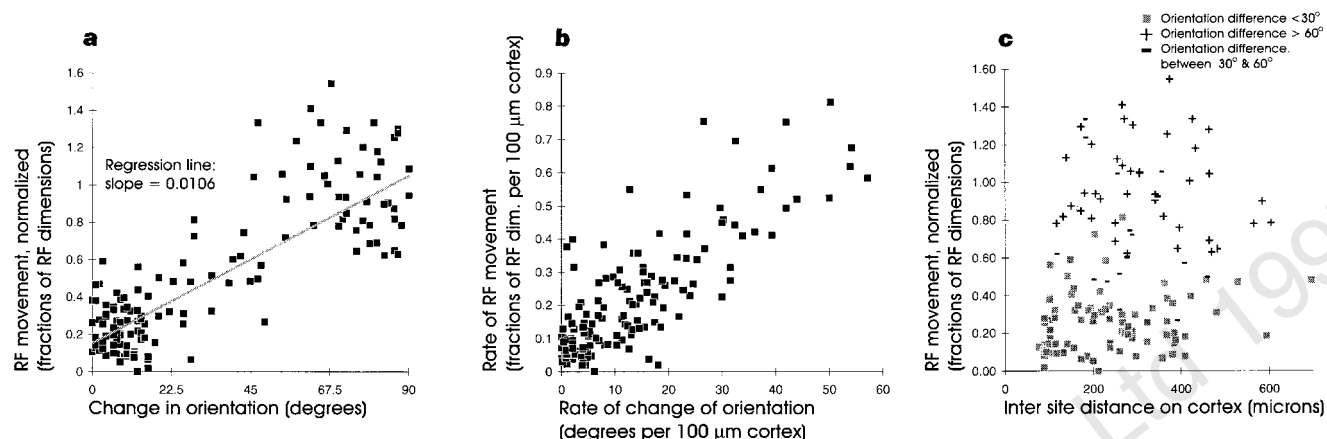


Figure 2 **a**, RF shifts are linearly proportional to orientation shifts over the entire population; graph of the movement of RF centres between neighbouring recording sites (for example, Ref. to 1, 1 to 2, 2 to 3, in Fig. 1d) as a function of the corresponding change in orientation preference. RF centre-to-centre distances, measured in degrees, were divided by the mean of the diameters of the two RFs, thus expressing RF movement in fractions of RF dimensions. **b**, Rate of RF movement is proportional to rate of change of orientation. Same data as in **a**, but divided, on each axis, by the cortical distance (in units of 100 μm) separating the pair of recording sites. This gives the rate of change of RF position per 100 μm of cortex, as a function of the rate of change of orientation per 100 μm of cortex. **c**,

There is no systematic bias in measurement of cortical positions. The same data as in **a**, replotted, show that RF shift is not correlated with inter-site distance ($R^2 = 0.06$). Further, RFs of similar orientation cluster together whereas RFs of dissimilar orientations are non-overlapping over a large range of cortical distances. To show the clustering, data points are grouped together by orientation shift; for example, the group '<30°' includes all RF pairs that differed by less than 30° in orientation. The sets of '<30°' and '>60°' data points form distinct populations in their associated RF shifts. RF pairs in the '<30°' group show high RF overlap at cortical separations out to 500 μm , whereas RF pairs in the '>60°' show little RF overlap even at cortical separations ~100 μm .

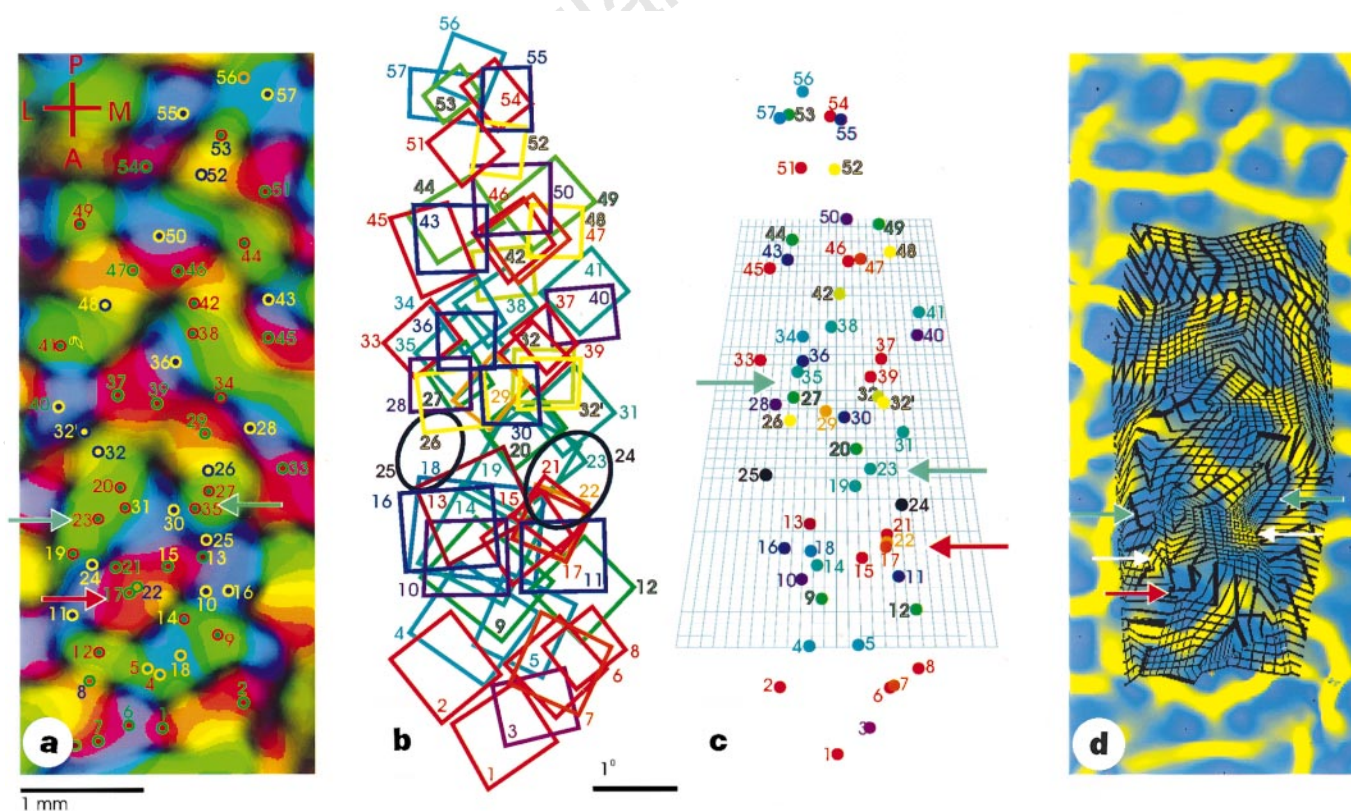


Figure 3 The two-dimensional map of visuotopy on V1 shows systematic local distortions that reflect inhomogeneities in the map of orientation columns. **a**, Optical map of orientation columns showing electrode recording sites. The green and red arrows point to recording sites whose RF centres are identified in **c**. **b**, The corresponding RFs. All RFs were mapped with respect to a fixed reference RF. RFs are colour-coded for orientation as in Fig. 1. **c**, The centres of the same RFs as in **b**, similarly colour-coded. Compare the distributions of two clusters of green RF centres (green arrows), and the cluster of red RF centres (red arrow) with the corresponding electrode recording sites (green and red arrows respectively in **a**.

Note, for example, the pairs of corresponding cortical sites 19 and 21 or 13 and 35 that give widely separated RFs.) The superimposed grid incorporates the average magnification factor that changes smoothly with eccentricity. **d**, The grid shown in **c**, mapped back to the cortical surface with the transform that maps RF centres smoothly back to their cortical recording sites. The grid is shown against the magnitude of the local gradient of orientation, colour-coded blue to yellow, scaled so that blue represents 0 and the brightest yellow indicates regions of most rapid local change of orientation (76.5° per 100 μm of cortex). Note that the experiment illustrated in this figure is in addition to those included in the population plots in Fig. 2).

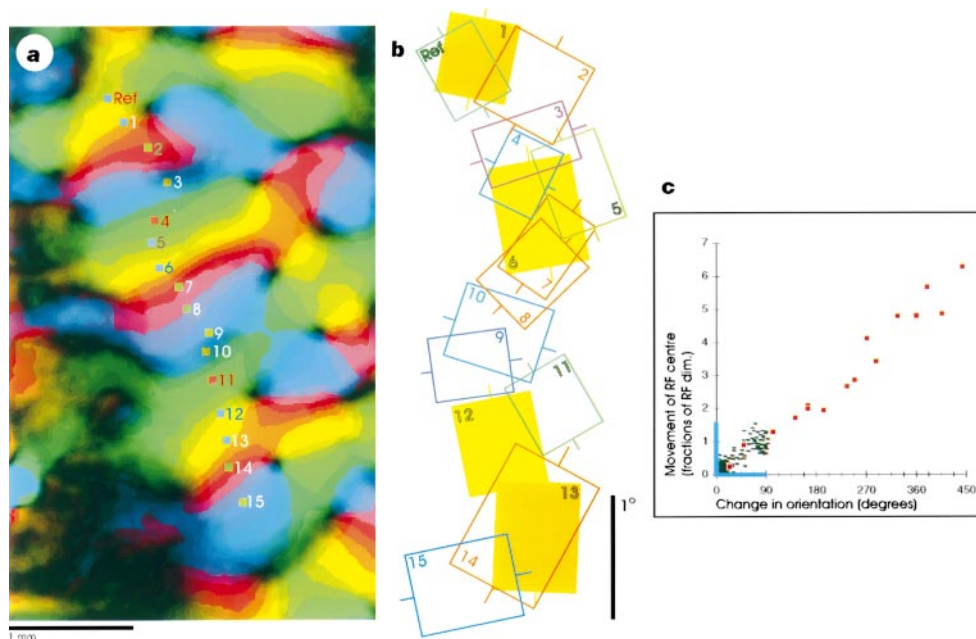


Figure 4 RFs move rapidly (~ 2 RF diameters per 180° of orientation shift) along any sequence of cortical recordings. **a, b**, Optical image and RFs over a uniformly changing sequence of orientation preference. **c**, Plot of RF shift against orientation shift for each RF with respect to the reference RF in **b** (regression fit: slope = 0.0140 RF diameters per degree (Table 1)). Data from Fig. 2a – where orientation shifts extend only to 90° for near neighbours – are replotted in **c** for comparison (highlighted in blue along the axes). Note that the experiment illustrated in this figure is in addition to those included in the population plots in Fig. 2.

tests reinforced our finding that RFs of similar orientation preference cluster together, whereas RFs of orthogonal orientations are non-overlapping (Fig. 2c), over a large range of cortical distances. To test against the null hypothesis that our results (Fig. 2a,b) could be accounted for by a linear visuotopic map with random scatter, we simulated sets of RF positions based on this null hypothesis (see Methods, 'Monte Carlo simulations, 1'). The simulated data, analysed as in Fig. 2a,b, gave correlation coefficients close to zero and showed none of the trends apparent in the real data (Table 1), making it highly improbable ($P < 10^{-7}$) that a linear map of visuotopy with additional scatter could lead to the observed relations between RF shift and orientation shift. In a separate check of the influence of measurement uncertainty on our results, we simulated data by adding realistic scatter to our real data (see Methods, 'Monte Carlo simulations, 2'). These simulated data gave slopes and correlation coefficients similar to our experimental results (Table 1), confirming that our findings are robust against measurement uncertainties.

The systematic modulation of the two-dimensional cortical visuotopic map, arising from the covariation of the rates of RF movement and orientation change (Fig. 2b), is illustrated in Fig. 3. A set of electrode recordings over the large area of mapped cortex (Fig. 3a) gave RFs (Fig. 3b) whose centres are mapped in Fig. 3c. Even though the recording sites (Fig. 3a) are roughly equally spaced over cortex, the corresponding RF centres are significantly clustered (Fig. 3a,c legend). The systematic distortion of visuotopy that results from this clustering is illustrated by transforming a grid from visual space on to the cortex, using the mathematical transformation that maps RF centres to their corresponding cortical recording sites (Fig. 3d; see Methods). Because the grid in Fig. 3c incorporates the change in average local cortical magnification over the recorded region of V1, modulations in the transformed grid in Fig. 3d reflect local modulations in magnification factor. Note the relative stretching of visuotopic dimensions over register of slowly changing orientation and relative compression in the regions where orientation changes rapidly. This match was quantified by cross-correlating the corresponding areal distortion with the orientation gradient and comparing with Monte Carlo simulations based on a linear map plus scatter. The comparison gave a probability of < 0.06 that a linear map plus scatter could account for the observed two-dimensional match between visuotopy and orientation gradient.

Our finding, of rapid RF movement (2 RF diameters per 180° change in orientation; Fig. 2a) was confirmed with an animal that

had a smooth orientation map with no singularities or reversals over 3 cycles of orientation (Fig. 4a). This allowed us to measure the orientation shift unambiguously, including intervening multiples of 180° , between the reference RF and each subsequent RF (Fig. 4b). The corresponding shifts in RF position from the reference are seen to be proportional to orientation shift, up to the end of the 3-cycle sequence (Fig. 4c), with regression slope matching Fig. 2a (Table 1). These results suggest the possibility that visual space might not be covered uniformly by all orientations, at least when V1 is not under the possible modulatory control of attention as in the alert animal¹⁰, and where RFs are not being dynamically modified^{11,12}.

The inhomogeneities in the visuotopic map in registration with fractures in the pattern of orientation columns lead to a number of questions concerning the perceptual consequences and possible mechanistic underpinnings of this pattern. One could ask, first, whether the observed distortion of visuotopy is merely a necessary consequence of the topology of visual cortex, of having to map the multiple dimensions of space, orientation and ocular dominance to the two-dimensional surface of cortex in a manner that is locally smooth. Theoretical studies indicate that this is not so: models of cortical development based on a principle of minimizing the lengths of lateral connections between cortical cells of similar RFs predict a pattern of distortion in retinotopy that is the precise opposite of what we have observed¹³. Our finding could thus have important implications for current concepts of cortical processing. Models of cortical development that try to minimize the differences in RFs of adjoining cells reflect the current thinking that local, lateral processing in V1 is effected through local circuits that interconnect neurons with similar or smoothly changing RFs. Such 'modules' of local processing are believed to populate the surface of V1 homogeneously. Our finding of correlated inhomogeneity in orientation and retinotopy suggests, instead, that cortical modules of local lateral processing could exhibit a wide range of functional relations. At cortical regions far from orientation singularities, such modules would link neurons with maximally similar RFs, whereas progressively closer to singularities such modules would link RFs with progressively greater separations in both RF position and orientation, thus leading to a corresponding range in the types of cortical calculations or comparisons performed in each module. □

Methods

Adult cats were anaesthetized, paralysed and prepared for electrical and optical recording from V1. To eliminate eye movement, eyes were fixed relative to the

stereotaxic apparatus by using a ring glued to the sclera. Stability of eye position was confirmed by monitoring RF positions with a fixed reference electrode through each experiment.

Optical imaging. The exposed brain surface was illuminated with light at 610 nm (interference filter, Oriel, 30 nm). Drifting gratings (1 cycle deg⁻¹, 1 Hz) were presented at each of eight orientations spanning 180°, filling the animal's visual space. For each orientation, video images of the brain surface (8 mm × 5 mm) were digitized and accumulated at video rates (Imager 2001 system from Optical Imaging, with Biscche CCD-6012P camera and Matrox IM-640 imaging board; imager was controlled by VDAQ software and images were analysed by using TVMIX as well as custom software). Each accumulated image was divided by the corresponding image at the orthogonal orientation to correct for nonspecific stimulus-driven changes in cortical reflectivity, and the resultant image was band-pass filtered by convolving with kernel of 2 gaussians, smoothing at 12 μm and high pass at 750 μm radius. The set of all filtered orientation difference images was combined vectorially, pixel by pixel, to calculate the strength and preferred orientation of the resultant optical signal. The computed resultant orientation value at each point is shown in the polar plot (Fig. 1a).

Electrophysiology. All RFs were multi-unit and, except where mentioned otherwise, were obtained within the first 300 μm (typically within the first 150 μm) of the cortical surface. For automated maps of RF pairs (Fig 1a–c), simultaneous grid maps were obtained for both RFs of a pair (site 1 is the reference site in Fig 1a–c) by using a computer-generated visual stimulus that consisted of a single test bar, flashed in pseudo-random sequence over an 8 × 8 grid of points spanning both RFs in visual space. For hand-mapped units, RF boundaries (and orientation preferences) were mapped with respect to the RF at a fixed reference electrode, by using a hand-held visual stimulator. RFs were plotted in a 'blind' procedure, where the person plotting RF boundaries was unaware of the cortical locations of recording sites. At each recording site, both the reference RF and the new RF were plotted on a fresh sheet of paper. This was done both to correct continually for any residual drift in eye position and to avoid being influenced by existing boundaries of either the reference RF or other RFs. (This process was followed for the data shown in Fig. 1d, and for 75 of the data points in Fig. 2. Earlier measurements were also made with respect to a reference RF, but not 'blind'. The results of these earlier measurements were statistically indistinguishable from the data obtained blind, and have been combined, therefore, in the population data, to give 70 of the 145 points in Fig. 2.) In quantitative analyses (Fig. 2), we used hand-mapped values of orientation preference. In cortical regions away from orientation singularities, these values differed from optically imaged values by less than 10° (r.m.s.) and the quantitative analyses with optically mapped orientation preferences gave results that were statistically indistinguishable from the results with hand-mapped values (see supplementary information). In a separate set of experiments, we measured the scatter in RF position and size in vertical penetrations. We measured RFs (again, with a fixed reference RF) at 50 μm intervals along penetrations down to 500 μm, at 24 sites in four experiments (see Fig. 1f for results).

Monte Carlo simulations. (1) To test against null hypothesis of linear map plus scatter, for each experiment we obtained the best fit for the linear transformation (magnification plus rotation) that mapped cortical coordinates on to experimentally obtained RF centres, by minimizing the sum of squares of residual 'scatter' in RF position. Simulated RF positions were then generated for (measured) cortical positions at the best-fit linear map from cortex plus random scatter (two-dimensional gaussian, s.d. = r.m.s. minimal scatter). (2) Effect of uncertainty in experimental values: for each experiment, Monte Carlo simulations started with real data and added scatter in cortical position (two-dimensional gaussian, radius 25 μm) and in RF position (0.1 × RF size; see Fig. 1f legend).

Two-dimensional map of distorted visuotopy (Fig. 3). The grid in visual space (Fig. 3c) was constructed as follows: for the set of recordings shown (Fig. 3a,c), average local cortical magnifications were measured and the change in this average value along the anteroposterior axis was determined (1.7:1, anterior:posterior). The grid was then constructed so that the size of grid elements in each row increased in a geometric progression (factor = 1.012) so as to give a final element size of 1.7 times the initial element size at the end of the sequence of grid rows. The mapping transform for RF centres was calculated as follows: for each recording site, the set of nearest-neighbour recording sites was identified; these vertices were connected to construct the set of nearest-

neighbour triangles tessellating the cortical surface. The vector displacement required to map each triangle vertex (electrode recording site) to its corresponding RF centre was calculated and the projections of the cortical triangles on visual space were determined. The inverse of this transform was then used to map an image of the grid in visual space back to the cortical surface. The points in the interior of each triangle in visual space were mapped by the reverse affine transform back to the cortical surface, carrying with them included segments of the visual grid. The local areal compression factor (deg²mm⁻²) was then calculated as follows: for each point on a regular grid on the cortex, the square defined by the nearest neighbouring lattice points was projected back on visual space, the ratio of the (transformed) square in visual space to the square on cortex was calculated, and the average subtracted.

Gradient of orientation. For the two-dimensional surface defined by orientation values on the cortex, the local tangent was calculated (deg per 100 μm of cortex); Fig. 3d shows the magnitude of this gradient vector. To calculate cross-correlation values, this scalar magnitude was multiplied, pixel by pixel, by the areal compression factor, and averaged over the area of the transformed grid.

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GABA in the mammalian suprachiasmatic nucleus and its role in diurnal rhythmicity

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Mammals manifest circadian behaviour timed by an endogenous clock in the hypothalamic suprachiasmatic nucleus (SCN)¹. Considerable progress has been made in identifying the molecular basis of the circadian clock^{2,3}, but the mechanisms by which it is translated into cyclic firing activity, high during the day and low at

night, are still poorly understood. GABA (γ -aminobutyric acid), a common inhibitory neurotransmitter in the central nervous system, is particularly densely distributed within the SCN, where it is located in the majority of neuronal somata^{4,5} and synaptic terminals^{6,7}. Using an *in vitro* brain-slice technique, we have now studied the effect of bath-applied GABA on adult SCN neurons at various times of the day. We find that GABA acts as an inhibitory neurotransmitter at night, decreasing the firing frequency; but during the day GABA acts as an excitatory neurotransmitter, increasing the firing frequency. We show that this dual effect, which is mediated by GABA_A receptors, may be attributed to an oscillation in intracellular chloride concentration. A likely explanation is that the amplitude of the oscillation in firing rate, displayed by individual neurons, is amplified by the dual effect of GABA in the SCN's GABAergic network.

There is considerable evidence indicating that GABA plays a pivotal role in circadian time-keeping, which is regulated by the firing rate of neurons in the SCN. First, there is extensive GABAergic innervation of the SCN (Fig. 1a, b)⁴⁻⁹. Moreover, cultured slice explants of the SCN, in which virtually all extrinsic inputs have degenerated, nevertheless retain a rich intrinsic GABAergic component (Fig. 1b)¹⁰. The concept of GABAergic network within the SCN appears to be supported by further observations: in freshly prepared SCN slices, 62% of the bicuculline-sensitive synaptic potentials are blocked by tetrodotoxin (TTX) ($n = 3$; results not shown), whereas the mean amplitude decreases by only 40% (the amplitudes of the synaptic potentials show a quantal distribution and only the higher peaks are abolished by TTX) indicating that the presynaptic cells existed within the slice; direct stimulation of SCN neurons with glutamate gives rise to GABAergic synaptic potentials in other SCN neurons^{11,12}; and dissociated SCN neurons communicate with each other solely via GABAergic synapses¹³. Thus, it appears that the SCN is composed of a functional, densely packed GABAergic network. Second, GABA and GABA agonists can phase-shift the circadian clock¹⁴, whereas GABA antagonists block both drug- and light-induced phase-shifts¹⁵. Third, mice devoid of prion protein, revealing weakened GABAergic synapses¹⁶, also have altered circadian rhythms^{17,18}.

To investigate the role of GABA in generating circadian behaviour and resetting the circadian clock, we studied the physiological effect of this neurotransmitter on SCN neurons. Using freshly prepared hypothalamic slices we measured, extracellularly, the effect of bath-applied GABA on the firing rate of neurons in the SCN. The circadian rhythm of firing rate of SCN neurons is well documented, the rate being high (8–10 Hz) during the day and low (2–4 Hz) at night^{19,20}. During the day, GABA at concentrations of 50–500 μ M reversibly increases the firing rate of SCN neurons (Fig. 2a), but at night GABA causes a reversible decrease in firing rate (Fig. 2b). Both the increase and decrease in firing rate are dose-dependent (Fig. 2c, d). Up to a concentration of 500 μ M, a monotonic relationship between the change in firing rate and the GABA concentration was usually observed. Both excitation (Fig. 2e) and inhibition (Fig. 2f) were blocked by picrotoxin or bicuculline, suggesting that both responses are mediated by GABA_A receptors, known to be coupled to chloride channels. Occasionally, the presence of the blocker itself has a reverse effect on the spontaneous firing: in 63% of the cases, where GABA increases the firing rate, bicuculline decreases it ($n = 8$) and vice versa ($n = 16$). The distribution of the responses (Fig. 2g) shows that during the day most of the neurons (71%) were excited by GABA application, whereas during the night most of the neurons (56%) were inhibited.

The effect of GABA on the activity of SCN neurons has been studied by several groups²¹⁻²³, showing that the most common response is a decrease in firing rate. In these studies, the excitatory response to GABA could have been masked by the use of high chloride concentrations in the extracellular pipette, which enforce inwardly directed chloride currents.

To reveal the mechanism by which the inhibitory neurotransmitter GABA acts as an excitatory agent, we used whole-cell patch recording to analyse the voltage across the membrane and its responses to current pulses before and during GABA application. In all neurons tested, GABA (100 μ M) increased the membrane conductance up to nine-fold (3.6 ± 2.7 nS, mean $\pm$ s.d., $n = 41$), independently of the time of the day ($P > 0.3$ unpaired *t*-test; Fig. 3e). This response was accompanied by a change in membrane potential which was correlated with the chloride concentration in the patch pipette ($[Cl^-]_p$). When low $[Cl^-]_p$ was used (2 mM),

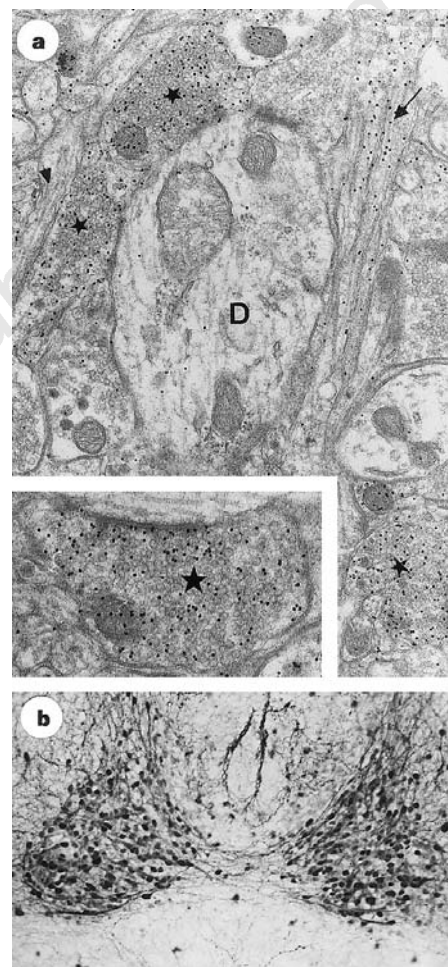


Figure 1 Immunocytochemical evidence for the extensive GABAergic innervation in the rat SCN. **a**, A typical field in the neuropil of the SCN, after post-embedding immunogold labelling of the GABA antigen. A large unlabelled proximal dendrite (D) is seen, onto which two GABA-immunoreactive terminals (asterisk) and two unlabelled terminals impinge. At the lower right part of the field is another labelled axo-dendritic GABAergic terminal (asterisk). At the upper right the concentration of gold particles (arrow) indicates a GABAergic neurite; an unlabelled neurite is seen at the upper left (arrowhead). Inset (lower left) is a larger magnification of a GABAergic terminal (large asterisk); note the gold particles are primarily over synaptic vesicles and mitochondria. Counts from our electron micrographs, derived from multiple levels within the SCN, showed that 40–70% of synaptic terminals per field were GABA-immunoreactive. **b**, Extensive GABA immunoreactivity in 3-week-old slice explant culture of the SCN. Note the numerous GABA-immunoreactive somata throughout the bilateral SCN, embedded in a dense web of immunoreactive processes. This GABAergic innervation is assumed to be of local origin, because the isolated explant retains few, if any, extrinsic GABAergic inputs. However, possible synaptic reorganization *in vitro* should be taken into account. Magnifications: **a**, $\times 20,500$; inset, $\times 40,000$; **b**, $\times 250$.

GABA induced hyperpolarization of up to 20 mV (Fig. 3a). In contrast, using 25 mM $[\text{Cl}^-]_p$ induced depolarization of the same magnitude (Fig. 3b). As with the firing rate, the voltage response to GABA was blocked by bicuculline (not shown). This, in addition to the dependence of the voltage response on $[\text{Cl}^-]_p$, further supports the possibility that GABA_A receptors are involved in the responses of SCN neurons to GABA.

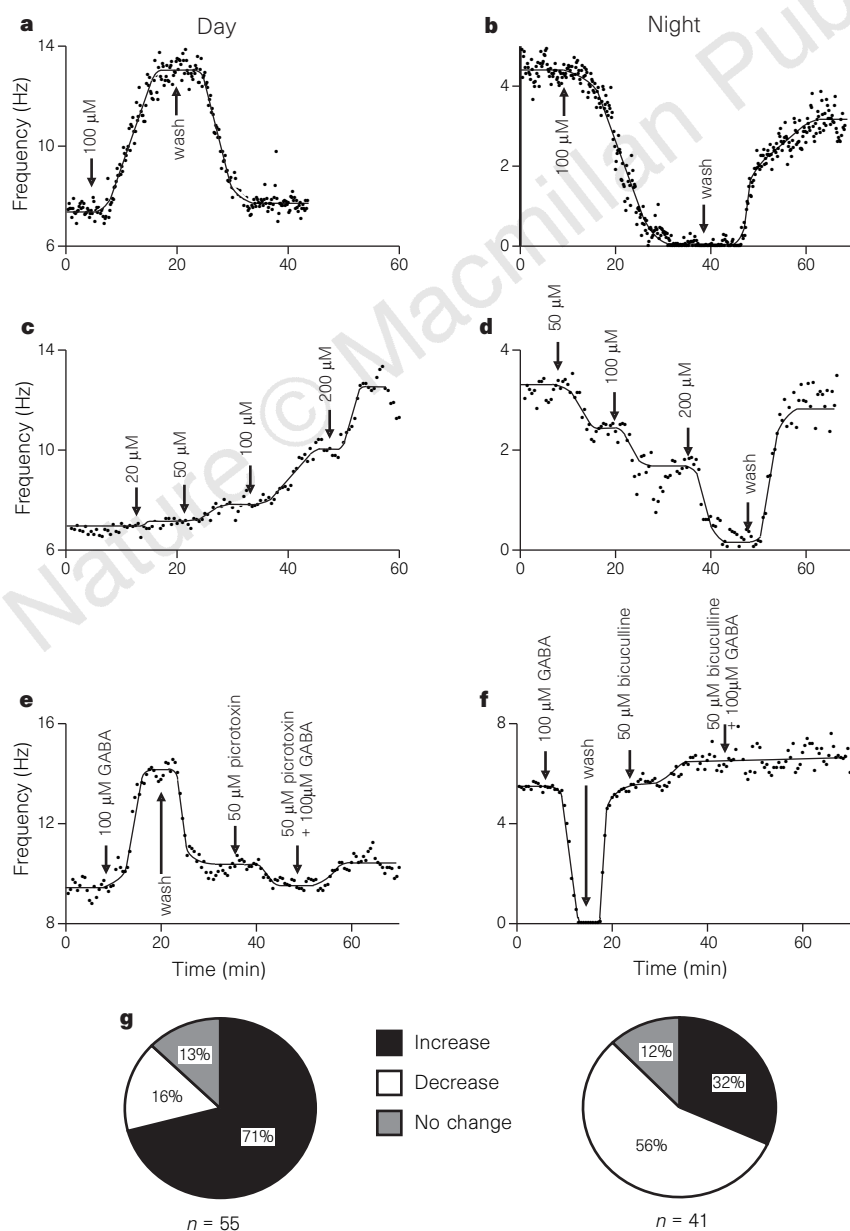
The experimentally determined reversal potential of the GABA response depends on $[\text{Cl}^-]_p$ (Fig. 3c, d), but is independent of the time of day ($P > 0.4$; Fig. 3f) for any given $[\text{Cl}^-]_p$. At 2 mM, an average reversal potential of -60 ± 5 mV ($n = 18$) was calculated for day and night animals. By contrast, a reversal potential of -31 ± 5 mV ($n = 22$) was calculated for $[\text{Cl}^-]_p$ of 25 mM. The difference in the measured reversal potential (29 mV) is smaller than expected from a 12.5-times change in $[\text{Cl}^-]_p$ (~ 60 mV). This difference is due partly to the permeability of bicarbonate and partly to the large influx of chloride during GABA application under low-chloride-concentration conditions²⁴. The absence of diurnal changes in measured reversal potential is related to the fact that in the whole-cell patch technique the intracellular ion composition is determined by the content of the patch pipette. Thus, by using the whole-cell patch technique we have overridden the endogenous

variations in cytoplasmic composition that occurred during the diurnal cycle.

An excitatory effect of GABA is often found in immature neurons of various origins^{25,26}, and has also been demonstrated in some mature neurons²⁷. The usual explanation for this phenomenon is reversal of chloride gradients, although bicarbonate permeability of the GABA_A channel and altered GABA receptors have also been suggested.

The hypothesis emerging from our results is that high intracellular chloride concentration ($[\text{Cl}^-]_i$) during the day induces an excitatory response to GABA, whereas low $[\text{Cl}^-]_i$ at night results in an inhibitory response. We tested this under conditions that enabled a glimpse of the original intracellular chloride concentration. These conditions exist during the first few seconds after breaking the cell membrane. Most of the spontaneous synaptic potentials recorded from SCN neurons are blocked by bicuculline (Fig. 4a, b) and have a reversal potential of about -30 mV (Fig. 4d; mean 31 ± 6 mV, $n = 5$; $[\text{Cl}^-]_p = 25$ mM), which is similar ($P > 0.5$, paired t -test) to the reversal potential of the GABA response (Fig. 4e). Both reversal potentials depend on the chloride concentration gradient. Thus, the change in amplitude of the spontaneous synaptic potentials that occurs after breaking the cell membrane should reflect the change in

Figure 2 GABA acts as an excitatory neurotransmitter during the day and as an inhibitory neurotransmitter at night. The data are presented in the form of firing rate as a function of time. **a, b**, Time course of the increase (**a**) and the decrease (**b**) in firing rate of SCN neuron in response to GABA (100 μM) application during day and night, respectively. Each point represents the mean frequency calculated over 30 s. Times for changing solutions and concentrations are marked on the plots. The continuous lines were drawn by hand to emphasize the time course of the changes. **c, d**, both the excitatory (**c**) and inhibitory (**d**) responses to GABA are dose-dependent. **e, f**, Both excitation and inhibition are blocked by picrotoxin or bicuculline. Note that in the presence of blocker, 100 μM GABA did not alter the firing rate of the neuron. **g**, Distribution of the neuronal responses to GABA during day and night. The response was calculated at steady-state conditions over a period of at least 5 min.



$[Cl^-]_i$ caused by equilibration with the electrode content. To analyse these changes quantitatively, we used the standard deviation (s.d.) of the membrane voltage. As shown in Fig. 4c, the variation in membrane potential, which mainly reflects synaptic activity, decreased with membrane depolarization. The dependence of the s.d. on the membrane potential (Fig. 4d, circles), matched that of synaptic activity amplitude (Fig. 4d, squares). This indicates the validity of using the s.d. as representative of the amplitude of synaptic events.

With 2 mM chloride in the patch pipette, the amplitude of the synaptic potentials and the s.d. of the membrane voltage decreased significantly during the first few seconds after breaking the cell membrane (Fig. 5a), indicating that the original $[Cl^-]_i$ was higher

than 2 mM. However, with 25 mM chloride in the pipette, an increase in the amplitude of the synaptic potentials was invariably observed (Fig. 5b), indicating that the original $[Cl^-]_i$ was lower than 25 mM. Using the s.d. analysis, the changes in amplitude of the spontaneous synaptic potentials were estimated and compared at various times of the day with $[Cl^-]_p = 15$ mM. The amplitude of the synaptic activity decreased (Fig. 5d) in 70% of the neurons from day animals (Fig. 4e). By contrast, an increase in amplitude of synaptic potentials (Fig. 5c) was observed in most (53%) of the neurons from night animals (Fig. 5e). Thus, it appears that during the day the intracellular chloride concentration in most of SCN neurons is higher than 15 mM, whereas during the night it is lower than 15 mM. The different distributions of neurons according to the

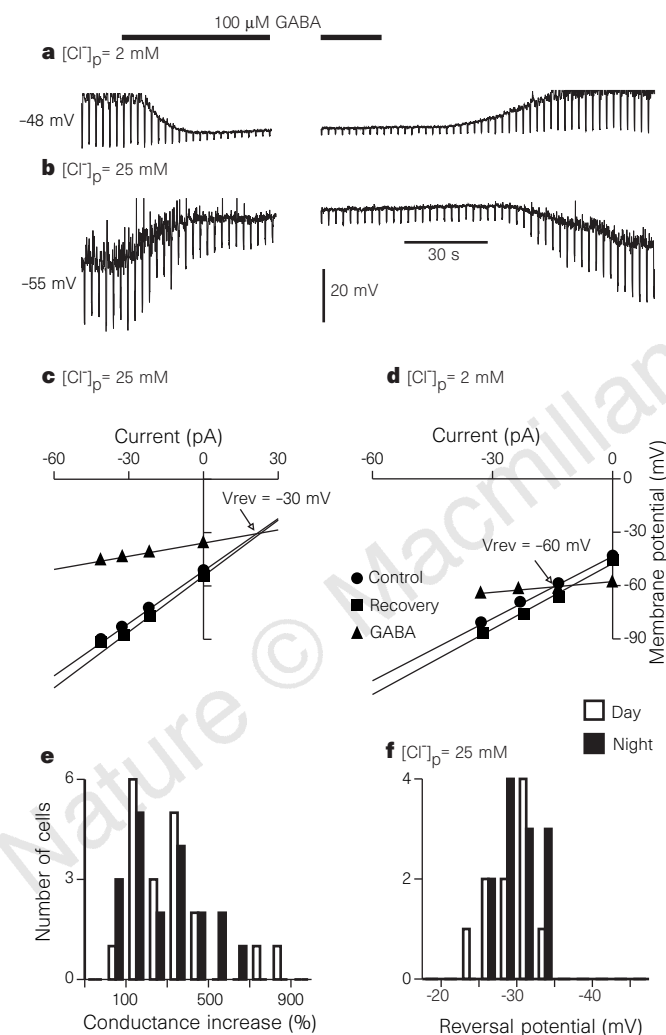


Figure 3 Neither the conductance change nor the reversal potential of the GABA response depend on the time of the day when the whole-cell patch technique was used. **a, b**, The membrane potential and the voltage response to hyperpolarizing current pulses (10 pA), before, during (bar) and after GABA application in day animals. The intracellular solution ($[Cl^-]_p$) contained 2 mM and 25 mM chloride in **a** and **b** respectively. The resting potential is indicated on the left of the traces. Action potentials in **a** were truncated. **c, d**, Current-voltage relationships, from the same experiments as in **b** and **a**, respectively, before (circles), during (triangles) and after (squares) GABA application. The change in slope and the intersection of the lines were used to calculate the conductance change and the reversal potential (V_{rev}) correspondingly. **e, f**, Distribution of the conductance change and reversal potentials of the GABA response, during day (white bars) and night (black bars). The reversal potential was calculated from experiments in which 25 mM of $[Cl^-]_p$ was used and conductance changes were calculated for all experiments.

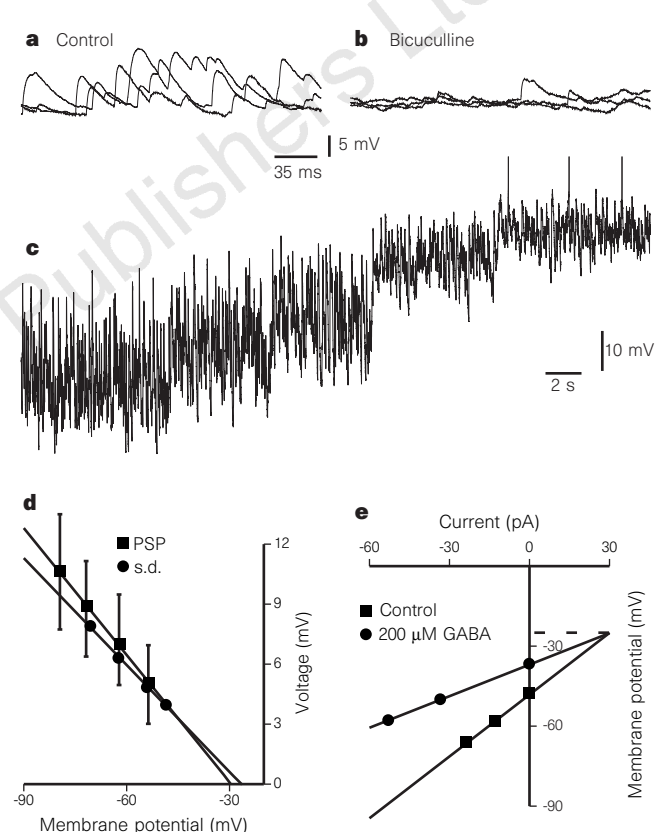


Figure 4 Most of the spontaneous synaptic potentials are GABAergic, and may be used to estimate the intracellular chloride concentration. **a, b**, Bicuculline (50 μ M) blocks most of the spontaneous synaptic potentials. Each panel shows three superimposed traces before addition (**a**) and in the presence (**b**) of bicuculline. **c**, Slow rate recording of the fluctuations in membrane voltage at different membrane potentials. The membrane potential was set by intracellular injection of d.c. current. **d**, Both the standard deviation (s.d.) and the mean amplitude of the postsynaptic potentials (PSP) depend on the membrane potential. A reversal potential of -30 mV and -28 mV was calculated, respectively. Bars represent the standard error. **e**, In the same cell, the calculated reversal potential of the GABA response was -28 mV.

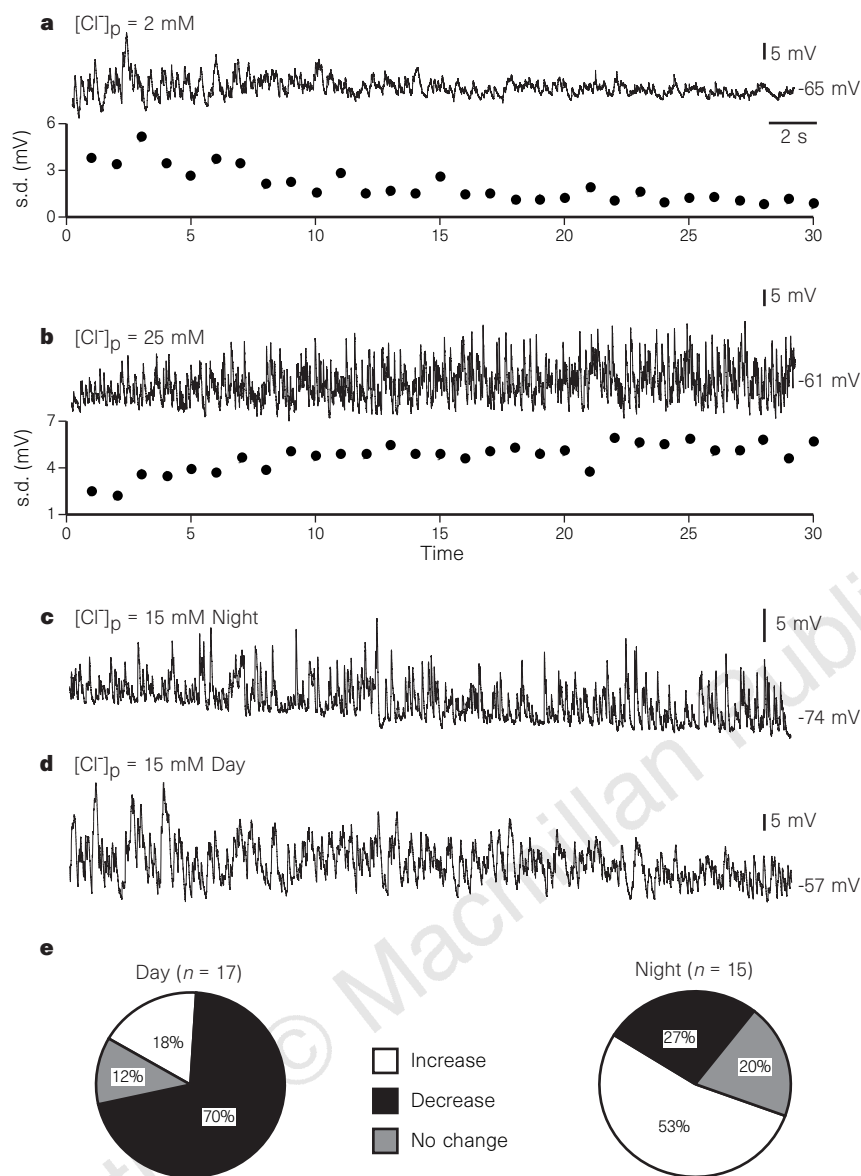


Figure 5 Postulated diurnal alterations in $[Cl^-]_i$ revealed by analysing the amplitude of the spontaneous synaptic potentials. **a**, Upper trace, continuous recordings of membrane potential during the first 30 s after breaking the cell membrane (in day animal). The patch pipette contained 2 mM chloride. Negative d.c. current was injected to prevent firing of the neuron and thus depolarizing synaptic potentials were always recorded. The mean membrane potential is indicated on the right of the trace. Lower trace, standard deviation of the membrane voltage during this period (see Methods). **b**, As in **a**, but for 25 mM chloride. **c**, **d**, As in **a**, for 15 mM of chloride in night and day animals respectively. **e**, Distribution of neurons according to changes in amplitude of synaptic potentials during the first 40 s after breaking the cell membrane. The patch pipette contained 15 mM chloride. The change in amplitude was calculated from the s.d. of the membrane voltage (see Methods).

calculated change in amplitude of synaptic potentials (Fig. 5e) is similar to that observed for the calculated firing rate (Fig. 2g).

These results indicate that the intracellular chloride concentration in SCN neurons undergoes diurnal oscillations, that is, high concentrations prevail during the day and low concentrations at night. SCN neurons may possess a cyclically modulated ion pump that determines the intracellular chloride concentration. A diurnal oscillation in $[Cl^-]_i$ has two immediate implications. First, with regard to the activity of the SCN's neuronal network, such a process would by itself increase the magnitude of the diurnal changes in firing rate, that is, the dense GABAergic local circuits would act as a positive feedback. At night, when neurons fire at low rates, GABA would act to decrease the firing further, but during the day, when GABA acts as an excitatory agent, the already high firing rate would be augmented. Although a diurnal change in $[Cl^-]_i$ could generate the diurnal variation in firing rate by itself, provided that the neurons are tonically active, it should be noted that isolated neurons exhibit circadian oscillation in firing rate¹³. Second, this new concept, that the intracellular ion composition can be a functional and modifiable physiological factor that governs the excitability of the nervous system, may account for the changes in level of excitability, as well as in susceptibility to epileptic discharge, that are known to occur during specific diurnal periods. □

Methods

Animals and brain slices. For electrophysiological recording fresh brain slices were derived from young (3–8 weeks) Sabra rats that had been entrained to a 12/12 light/dark cycle for at least 2 weeks. Coronal hypothalamic slices (300–500 μm) were prepared as described¹⁰, at zeitgeber time (ZT) 2–4 and ZT 15–17 for day and night experiments, respectively. Following stabilization of the slices in aerated physiological solution, experiments on day animals were performed during ZT 4–9 and on night specimens during ZT 17–21.

Physiology. The physiological solution, aerated with 95% O_2 and 5% CO_2 , was composed of (in mM): NaCl 120; KCl 5; $MgSO_4$ 1.3; KH_2PO_4 1.2; $NaHCO_3$ 26; $CaCl_2$ 2.4; glucose 20. The submerged-slice technique was used throughout. The current of single unit activity was extracellularly recorded by an AXO-PATCH-1D amplifier in the voltage clamp mode at 36 °C. The glass pipette was filled with physiological solution and had a d.c. resistance of 4–8 M Ω . The neurons were randomly sampled from various areas in the SCN and their firing rates were continuously estimated from the average interspike interval calculated every 0.5–2 min. GABA (final concentration, 10–500 μM), bicuculline (20–50 μM) and picrotoxin (50–100 μM) were bath-applied via the physiological solution. The response to GABA was determined by calculating the average firing rate during 5–10 min (~10 independent measurements) of steady-state conditions before and during GABA application. A standard *t*-test was used to estimate the significance of the difference between the measurements

made before and during GABA application. Changes of less than 5% were considered as 'no change', even if the *t*-test showed significance.

The whole-cell patch technique was used for intracellular recordings (AXOCLAMP-2A in current clamp mode at room temperature). The intracellular solution was composed of (in mM): potassium gluconate 120; NaCl 24; CaCl₂ 0.5; Mg-ATP 5; EGTA 5; HEPES 10. The chloride concentration was varied between 2, 15 and 25 mM by replacing the NaCl with sodium gluconate; pH was set to 7.2 with KOH.

The slope of the current-voltage relationship (*I*-*V* curve) was drawn from the voltage response to 250–350 ms negative current pulses of different intensities (10–60 pA). The conductance change in response to GABA application was calculated from the slopes of *I*-*V* curves in the presence or absence of GABA. The intersection point of these curves was used to estimate the reversal potential of the response to GABA (Fig. 3c, d).

The change in amplitude of the synaptic potentials during the first several tens of seconds after breaking the cell membrane was estimated by calculating the s.d. of the membrane voltage, digitized at a rate of 1 kHz, within a time window of 1 s (Figs 4, 5). The s.d. of the first second was compared to the s.d. at steady-state conditions (after at least 40 s). A change of less than 5% was considered as 'no change'.

Morphology. For immuno-ultrastructural observations, adult rats were anaesthetized with intraperitoneal sodium pentobarbital and, following a brief saline wash of the blood system, were perfused with fixative containing 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.0. Vibratome sections containing the SCN were post-fixed in 1% osmium tetroxide and 1.5% potassium ferricyanide in 0.05 M cacodylate buffer, and processed for electron microscopy²⁸. On ultrathin sections, post-embedding immunogold labelling was with anti-GABA antibodies (Somogyi code no. 9) and protein-A gold (15 nm) as described²⁸.

Organotypic slice explant cultures of the SCN were derived from neonate (9–12 days) Sabra rats as described¹⁰. After 2–3 weeks *in vitro*, cultures were immersed in fixative containing 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.0, before pre-embedding and immunoperoxidase labelling with anti-GABA antibodies. Briefly, following incubation in primary antiserum, cultures were reacted with the avidin-biotinylated peroxidase complex (Vector), and immunoreactivity visualized with diaminobenzidine tetrahydrochloride. Cultures, whole-mounted on subbed slides, were dipped in dilute aqueous osmium tetroxide, dehydrated in ethanol, and coverslipped.

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The Rx homeobox gene is essential for vertebrate eye development

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Development of the vertebrate eye requires a series of steps including specification of the anterior neural plate, evagination of the optic vesicles from the ventral forebrain, and the cellular differentiation of the lens and retina. Homeobox-containing genes, especially the transcription regulator *Pax6*, play a critical role in vertebrate and invertebrate eye formation. Mutations in *Pax6* function result in eye malformations known as *Aniridia* in humans and *Small eye* syndrome in mice^{1–3}. The *Drosophila* homologue of *Pax6*, *eyeless*, is also necessary for correct invertebrate eye development, and its misexpression leads to formation of ectopic eyes in *Drosophila*^{4,5}. Here we show that a conserved vertebrate homeobox gene, *Rx*, is essential for normal eye development, and that its misexpression has profound effects on eye morphology. *Xenopus* embryos injected with synthetic *Rx* RNA develop ectopic retinal tissue and display hyperproliferation in the neuroretina. Mouse embryos carrying a null allele of this gene do not form optic cups and so do not develop eyes. The *Rx* gene family plays an important role in the establishment and/or proliferation of retinal progenitor cells.

The *Rx* homeobox genes were first isolated from a cDNA library made from *Xenopus* animal-cap ectoderm induced by treatment with ammonium chloride. This treatment was chosen because it can activate transcription of genes involved in anterior head formation^{6–8}. The predicted amino-acid sequence of the *Xenopus* *Rx* genes shows that each gene contains a highly conserved paired-like homeodomain⁹ and octapeptide¹⁰ typical of this homeobox gene subfamily (Fig. 1a). They also contain a conserved motif in the carboxy-terminal end of their proteins. This domain is present in several paired-like homeobox genes, and the consensus sequence of

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Table 1 Overexpression of *Rx* in *Xenopus* embryos

Injection clone	Injection site	Pigment streaming	Retinal duplication	Neural tube hyperplasia	Neural tube branching/duplication
<i>Rx1</i>	dorsal	86% (113/132)	60% (88/146)	60% (34/57)	67% (10/15)
<i>Rx1</i>	ventral	33% (13/40)	48% (19/40)	13% (2/16)	—
<i>Rx1</i>	vegetal	0% (0/20)	0% (0/20)	0% (0/20)	—
<i>Pax6</i>	dorsal	0% (0/20)	0% (0/20)	0% (0/20)	0% (0/5)
<i>Otx2</i>	dorsal	0% (0/20)	0% (0/20)	0% (0/20)	0% (0/5)

primarily in the developing retina (Fig. 4b,c), whereas *Zrx3* is expressed primarily in the ventral forebrain (Fig. 4e,f), suggesting that during evolution there was a duplication and divergence of *Zrx* gene function. As a consequence, two of the genes are now active almost exclusively in the retina, whereas the third gene is active in the ventral forebrain. Their combined expression pattern encompasses the expression domains of the mouse and *Xenopus* *Rx* genes.

The conservation of expression pattern of *Rx* genes extends to invertebrates. The *Drosophila* *Rx* gene is expressed during early embryogenesis in the eye-disc primordia^{4,16} (Fig. 4g,h) and in a patch of cells in the anterior of the embryo corresponding to the clypeolabrum, in a region that gives rise to the epiphysis^{16,17}.

The expression pattern of the vertebrate *Rx* genes in early neurulae implies that they are involved in either the initial determination of the anterior region of the neural plate, the specification of the retinal progenitor cells, and/or in their subsequent proliferation. If *Rx* is indeed involved in any of these functions, then the overexpression or deletion of the *Rx* genes would be expected to have profound effects on eye development.

We have tested the effects of overexpression of *Rx* genes on normal eye development by injection of *Xrx1* synthetic RNA into 4–8-cell stage *Xenopus* embryos. As shown in Table 1, 86% of the embryos injected with *Xrx1* RNA into dorsal animal blastomeres developed ectopic retinal pigment epithelium (RPE) between the eyes and the neural tube. This tissue is most frequently present in the form of cells streaming from the eye towards the brain, but isolated patches of RPE can also be observed (Fig. 5a). This tissue will form only in the proximity of the anterior neural tube, suggesting that this region is providing a signal needed for retinal formation in addition to that of the *Rx* gene product.

As well as having this ectopic tissue, the RPE surrounding the sensory retina is often thicker than the RPE on the uninjected side (Fig. 5c,d), suggesting hyperproliferation of the pigment epithelium as a result of *Xrx* expression. The pigmented tissue reacts with antibodies against RPE (Fig. 5b) (ref. 18), demonstrating that *Xrx1* misexpression can cause ectopic retinal tissue formation. The ectopic RPE frequently connects the retina and neural tube, but this tissue is not simply a misplaced optic nerve or an optic nerve invaded by retinal pigment epithelium, as serial sections on these embryos showed the optic nerve to be in its usual ventral position (data not shown). Embryos injected with RNA into ventral animal blastomeres show less ectopic RPE development (33%) than those given dorsal injections. These data agree well with a previous observation that the ventral animal blastomeres produce significantly fewer descendants in the retina than the dorsal animal blastomeres¹⁹. Embryos injected in vegetal blastomeres showed no abnormal phenotype (Table 1). Superficially, the eye does not seem to be duplicated, although in cross-section it has the appearance of duplicated retinal layers enclosed within the RPE (Fig. 5c,d). The inward folding of the photoreceptor layer and protrusion outside the domain of the RPE (Fig. 5e) may result from the excessive production of differentiated retinal cells owing to an increased number of retinal progenitors. In support of this interpretation, we have observed cells that are morphologically similar to those at the ciliary margin ectopically located in the central retina, adjacent to the regions of ectopic RPE in *Xrx1*-injected embryos.

The region lying between the primary neural tube and the eye is

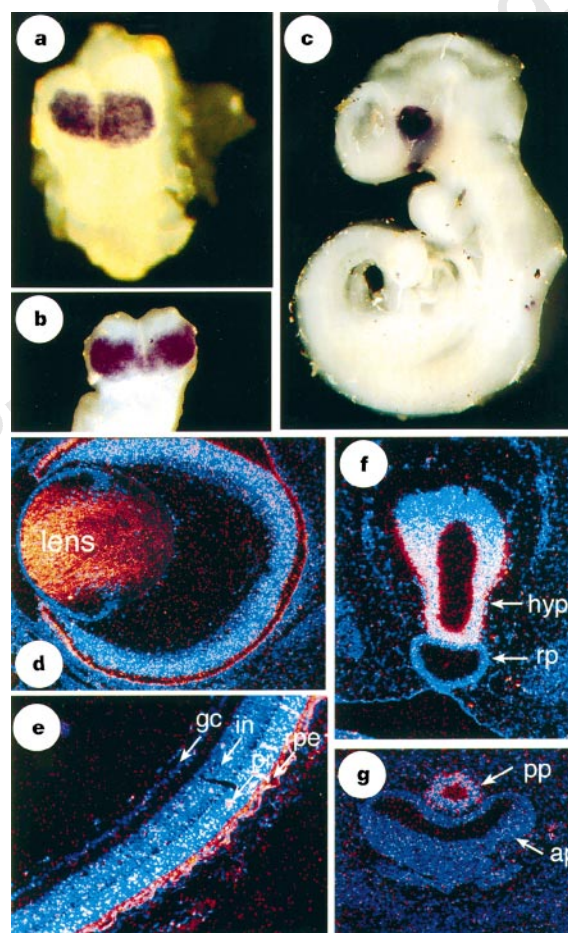


Figure 3 *In situ* hybridizations with *Mrx* probe. **a**, Whole-mount *in situ* hybridization showing expression in the anterior neural plate of E8.5 embryos. **b**, Localization to the presumptive optic area of E9.0 embryos. **c**, Expression in the optic cups and ventral forebrain of E10.5 embryos. **d**, Section of E15.5 mouse eye hybridized with ³⁵S-labelled *Mrx* riboprobe showing that expression is uniform within the neuroretina. **e**, By P6.5, *Mrx* is absent from ganglionic cells. **f**, Expression in the ventral hypothalamus at E10.5. **g**, Expression in the posterior pituitary at E12.5. Abbreviations: gc, ganglion cell layer; in, inner nuclear layer; pr, photoreceptor layer; rpe, retinal pigment epithelium; hyp, hypothalamus; rp, Rathke's pouch; ap, anterior pituitary; pp, posterior pituitary.

frequently altered, and has the histological appearance of a secondary neural tube (see Fig. 5c, arrow). The morphological consequence in the anterior neural tube of *Xrx* injection ranges from neural hyperplasia to branching and duplication of the neural tube. Embryos injected with *Pax6* and *Otx2* synthetic RNAs did not display such a phenotype (Table 1). Given the expression pattern and injection phenotype, it seems that overexpression of *Xrx* gene product results in an aberrant recruitment of cells towards anterior neural and retinal cell fates, or plays an important role in their subsequent proliferation. Elimination of *Rx* gene function would

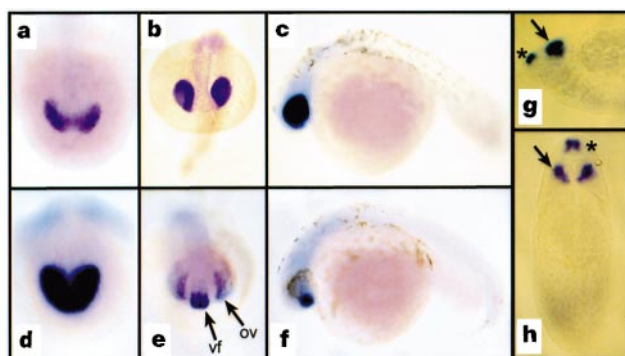


Figure 4 Whole-mount *in situ* hybridization to zebrafish (**a–f**) and *Drosophila* (**g, h**) embryos. **a**, Anterior view of *Zrx1* expression in the anterior neural plate at 10 somites. **b**, *Zrx1* expression in the retinal primordia at 20 somites (anterior view). **c**, *Zrx1* expression in the developing retina at 24 h. **d**, Anterior view of *Zrx3* expression localized to the presumptive retina and ventral forebrain at 5 somites. **e**, Anterior view of *Zrx3* expressed strongly in the hypothalamic region, but weakly in the developing retina; vf, ventral forebrain; ov, optic vesicle. **f**, *Zrx3* is restricted to the ventral forebrain at 24 h. **g**, *Drosophila Rx* expression in the head of a stage-10 embryo (5 h old). Anterior is to the left, dorsal is up. Arrow points to the eye-antennal disc primordia; asterisk marks the epiphysis. **h**, *Drx* expression in a 6-h embryo.

therefore be expected to result in a significant reduction of the eye field.

To test whether a loss of function of the *Rx* gene affects the eye phenotype, we generated a *Mrx* null mutant mouse, using targeted mutagenesis and embryonic stem-cell technology. The gene targeting strategy is shown in Fig. 6a. The homozygous newborn mice bearing the null allele of this gene have no visible eye structures, whereas mice heterozygous for the *Rx* mutation are apparently normal (Fig. 6b). The absence of eyes is even more obvious at E13.5 (Fig. 6c). The abnormal phenotype of these embryos is visible as early as E9.0 and E10.5 by a failure to form the indentations (optic sulci) that give rise to the optic cups (Fig. 6d,e), demonstrating clearly that *Mrx* function is required for eye formation from its initial stages.

As well as having eye defects, most of the embryos show a significant reduction of their brain structures (Fig. 6g). The mild phenotype consists of the absence of the ventral forebrain, but severely affected embryos have no forebrain at all and their mid-brain is also severely affected. These defects indicate that the early expression of *Mrx* in the presumptive forebrain and possibly part of the midbrain is essential for proper anterior neural development.

In summary, *Rx* genes are a conserved homeobox gene subfamily that are first activated in a uniform field in the anterior dorsal region of gastrula-stage vertebrate embryos. Their expression encompasses the entire presumptive eye field forms a single domain which only later splits into two lateral areas. As development proceeds, *Rx* expression correlates with the location of retinal stem cells, suggesting a fundamental role for these genes in the survival of retinal progenitor cells. The overexpression of *Rx* mRNA induces formation of ectopic retinal tissue and hyperproliferation of the neural retina in *Xenopus* embryos. The targeted knockout provides genetic evidence that the murine *Rx* gene is essential for eye formation. The *Mrx* null embryos are unique in their failure to form an optic vesicle, as even *Small eye* mutant embryos show optic vesicle formation, although it is

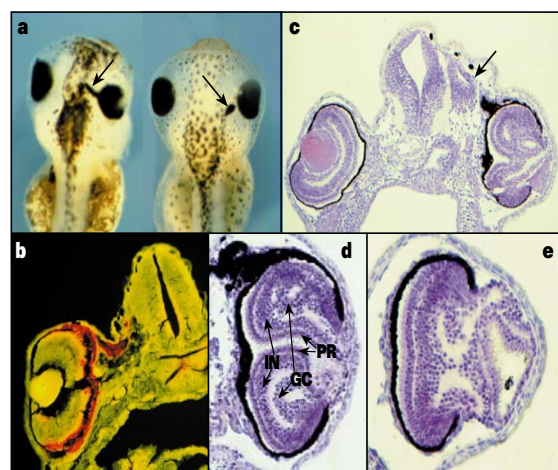


Figure 5 Embryos injected with *Rx1* RNA. **a**, Stage-41 embryos, displaying ectopic RPE connected to the eye (left arrow) and an isolated patch of ectopic RPE (right arrow). **b**, Cross-section of a stage-41 embryo reacted with antibodies against RPE, with ectopic pigmentation staining red. **c**, Cross-section of an embryo, showing duplication of the anterior neural tube (arrow), ectopic RPE formation, and a partial duplication of the retina. **d**, Cross-section through an eye displaying duplication of the retina and the presence of ectopic RPE; PR, photoreceptor layer; IN, inner nuclear layer; GC, ganglion cell layer. **e**, Cross-section through an eye showing protrusion of retinal tissue beyond the boundaries of the endogenous RPE.

abnormal morphologically^{20,21}. The mutant *Mrx* mice also lack anterior brain structures, demonstrating that the *Mrx* gene controls the size of the anterior head region. These results show that the *Rx* genes play a critical role in eye formation by regulating the initial specification of retinal cells and/or their subsequent proliferation.

Methods

Isolation of *Rx* homeobox genes. The NH_4Cl -induced library was constructed using poly(A)⁺ RNA collected from 400 animal-cap explants treated with 20 mM NH_4Cl in Holtfreter's buffer for 4 h. *Rx* homeobox fragments were identified using degenerate paired-class primers (RTHYPD and QVWFQNR) and used to screen a *Xenopus* stage-13 library²² for full-length clones. The mouse and *Drosophila Rx* homeoboxes were isolated using the degenerate primers DDEQPKKKH and PEVRVQVWFQN. The probes for *in situ* hybridization were generated by 5'-RACE reaction using homeobox sequences. The zebrafish *Rx* genes were cloned by degenerate PCR and full-length clones were isolated from neurula-stage (*Zrx3*) and postsomitogenesis-stage (*Zrx1* and *Zrx2*) libraries.

Embryos and *in situ* hybridization. *Xenopus* embryos were staged as ref. 23, and *Drosophila* embryos as ref. 17. *In situ* hybridizations followed standard protocols^{8,24,25}.

Microinjection and histology. Embryos shown in Fig. 4 were injected unilaterally at the 4- or 8-cell stage with 200 pg *Rx1* RNA into a dorsal animal blastomere. Injected tadpoles were grown to stage 41–42, embedded in JB-4, serially sectioned at 8 μm , and stained with haematoxylin and eosin. For antibody detection, the anti-RPE antibody XAR-1 was used at 1:10 dilution, and visualization used the Vector Labs A-B-C kit and VectorRed stain. Sectioning and staining of the mouse pups were performed by American Histolabs.

Targeted mouse deletion strategy. Flanking regions of 4.7 kb each from the *Mrx* locus were cloned into the XpNPT vector such that, upon targeting in embryonic stem cell, the 1.8-kb neomycin selection gene would replace a 2.7-kb region of the *Mrx* locus. This deleted region contains the 5'-portion of the *Mrx* transcript, including the protein initiation site and the N-terminal end of the

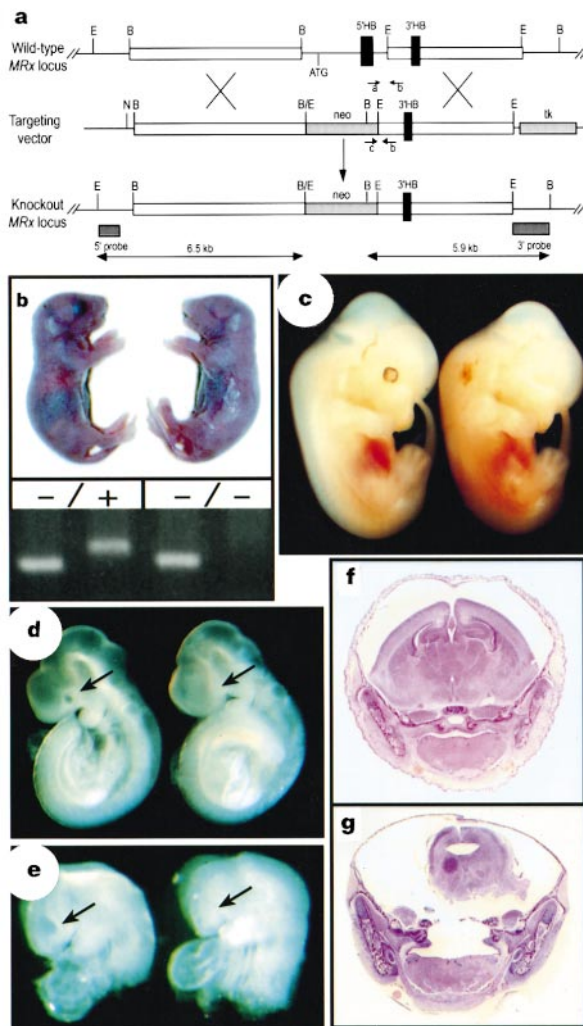


Figure 6 **a**, Schematic representation of *Mrx* targeted deletion strategy. E, *Eco*RI; B, *Bgl*II; N, *Not*I; HB, homeobox. **b**, Homozygous pups (right) are born without visible eye structures, whereas heterozygous pups (left) show no altered phenotype. PCR analysis of these pups show that the null (–/–) genotype correlates with the eyeless phenotype. **c**, Knockout embryos (right) show no eyes at E13.5. **d**, Knockout embryos (right) lack visible signs of optic cup formation (arrow) in E10.5 embryos. **e**, E9.0 null embryos (right) lack the optic depressions in the neural tube. **f**, Section through a heterozygous pup showing normal patterning of the forebrain. **g**, Section of a newborn anophthalmic homozygous pup displaying severe ablations of the forebrain and midbrain.

homeodomain. Tests on embryonic samples (see Fig. 6b) used PCR with two *Mrx* gene-specific primers and a neo-specific primer.

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Correspondence and requests for materials should be addressed to M.J. (e-mail: jamrich@al.cber.fda.gov). Genbank accession numbers for the homeobox genes are as follows: *Xenopus Rx1A*, AF001048; *Xenopus Rx2A*, AF001049; mouse *Rx*, AF001906; zebrafish *Rx1*, AF001907; zebrafish *Rx2*, AF001908; zebrafish *Rx3*, AF001909; *Drosophila Rx*, AF001910; and human *Rx*, AF001911.

The putative chaperone calmegin is required for sperm fertility

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The proper folding of newly synthesized membrane proteins in the endoplasmic reticulum (ER) is required for the formation of functional mature proteins. Calnexin is a ubiquitous ER chaperone that plays a major role in quality control by retaining incompletely folded or misfolded proteins^{1–5}. In contrast to other known chaperones such as heat-shock proteins, BiP and calreticulin, calnexin is an integral membrane protein^{1,6}. Calmegin is a testis-specific ER protein that is homologous to calnexin^{7–9}. Here we show that calmegin binds to nascent polypeptides during spermatogenesis, and have analysed its physiological function by targeted disruption of its gene. Homozygous-null male mice are nearly sterile even though spermatogenesis is morphologically normal and mating is normal. *In vitro*, sperm from homozygous-null males do not adhere to the egg extracellular matrix (zona pellucida), and this defect may explain the observed infertility. These results suggest that calmegin functions as a chaperone for one or more sperm surface proteins that mediate the interactions between sperm and egg. The defective zona pellucida-adhesion

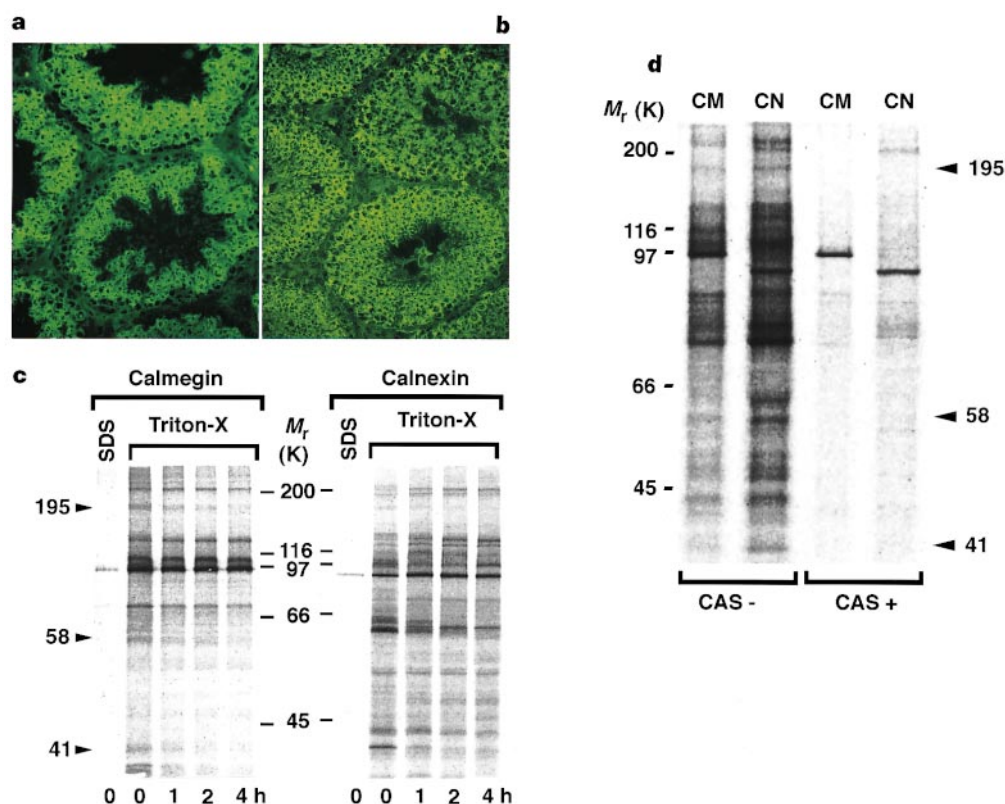


Figure 1 Calmegin and calnexin interact with nascent glycoproteins during spermatogenesis. **a, b**, Immunofluorescence staining of testicular sections with anti-calmegin or anticalnexin antibodies. Calmegin was not detected in the outermost layer (spermatogonia and Sertoli cells) or the innermost layer (elongated spermatids) of the seminiferous tubule, either while the ubiquitous expression of calnexin was evident (**a**) or during spermatogenesis (**b**). **c**, Both calmegin and calnexin transiently interact with nascent proteins in the testicular cells. Testicular cells were labelled for 30 min with ^{35}S -methionine and chased for

the indicated times (0–4 h). Cell extracts were immunoprecipitated with anti-calmegin or anti-calnexin antibodies. The immune complexes were resolved by SDS-PAGE. Arrowheads indicate nascent proteins (of M_r 195K, 58K and 41K) interacted with calmegin. When the cells were lysed in the presence of 1% SDS (denaturing condition) and were immunoprecipitated, a single polypeptide of calnexin and calmegin bands were observed, indicating that the antibodies were monospecific. **d**, CAS, a glucosidase inhibitor, abolishes the interaction of the calmegin and calnexin with nascent proteins. CM, calmegin; CN, calnexin.

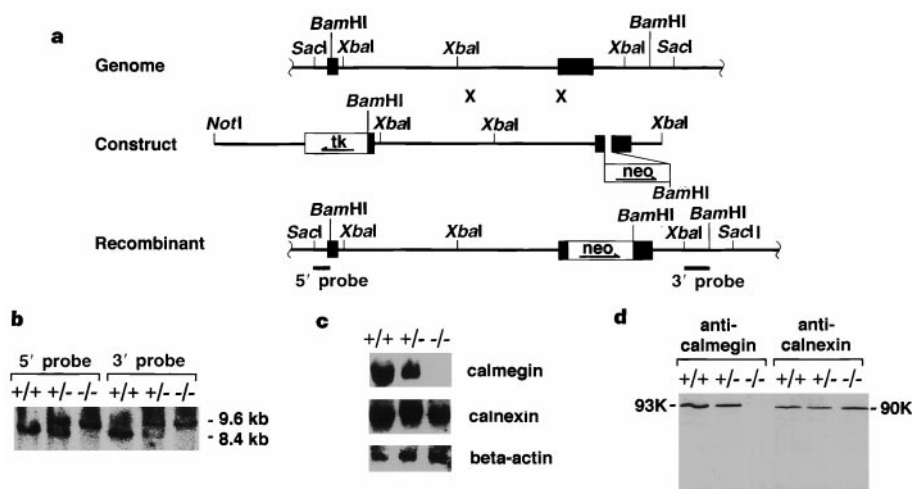


Figure 2 Targeted disruption of calmegin gene. **a**, Targeting vector was made by inserting a neomycin resistance (*neo*) gene into the second exon resulting in disruption of the calmegin ATG start codon. For negative selection, a herpes simplex virus thymidine kinase gene (*tk*) was introduced into the targeting construct. **b**, Hybridization of the 5'- and 3'-external probes with *SacI*-digested

genomic DNA yielded 8.4-kb (wild-type) and 9.6-kb (mutant) bands. **c**, Northern blot analysis of total testis RNA (20 µg) from wild-type, heterozygote and homozygote. **d**, Proteins (5 µg) from testicular cells were analysed by immunoblotting for calmegin and calnexin expression in mutant mice.

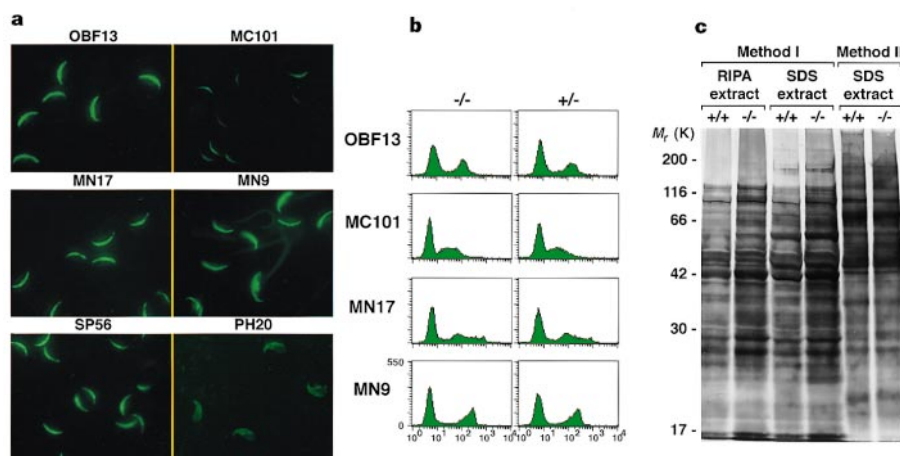


Figure 3 a, Sperm from calmegin-deficient and wild-type mice were stained with anti-acrosomal monoclonal and polyclonal antibodies (OBF13, MC101, MN17, MN9, sp56 and PH-20; see Methods). Staining patterns were the same as wild-type sperm (data not shown). **b**, Flow-cytometric analysis of preincubated sperm from calmegin $+/+$ and $+/-$ mice stained with anti-acrosomal antibodies (OBF13, MC101, MN17, MN9). The less intense peaks correspond to the 'negative'

population that appeared during incubation: 'bright' peaks represent the amount of each antigen contained in the sperm. **c**, The sperm surface was biotinylated and solubilized with RIPA or SDS and analysed by SDS-PAGE using avidin-peroxidase conjugate and chemoluminescence. We detected no difference in staining patterns between sperm from knockout and wild-type mice. Biotinylation was in EDTA-PBS (method I) or sodium bicarbonate buffer, pH 8.6 (method II).

Table 1 *In vitro* fertilization with sperm from calmegin-deficient mice

Genotype	Eggs with zona-penetrated sperm (%) [*]	Eggs with pronuclei (%) [†]
$+/+$, $+/-$	87/99 (88.8 $\pm$ 7.6)	65/75 (86.7 $\pm$ 8.6)
$-/-$	2/100 (1.3 $\pm$ 2.2)	0/72 (0)

Three independent experiments were performed.

^{*}Fertilization events were observed 1 h after insemination.

[†]Fertilization events were observed 8 h after insemination.

phenotype of sperm from calmegin-deficient mice is reminiscent of certain cases of unexplained infertility in human males.

Calmegin was first identified as a male meiotic germ cell-specific antigen⁸, and was later found to be a Ca^{2+} -binding protein localized on the ER membrane^{7,9}. Sequence analysis demonstrated that calmegin is highly homologous to calnexin, with 54% identity in amino-acid sequence, including two sets of characteristic sequence repeats necessary for Ca^{2+} binding^{7,9}. We determined whether calmegin functions as a calnexin-like molecular chaperone during spermatogenesis. In the testis, calmegin was expressed exclusively in germ cells of pachytene spermatocyte to spermatid stage⁸ (Fig. 1a), whereas calnexin was expressed throughout the stages (Fig. 1b). In the ER of somatic cells, calnexin associates transiently with nascent membrane and soluble glycoproteins of the secretory pathway and facilitates folding²⁻⁵. When calmegin was immunoprecipitated from testicular cells pulse-labelled with ³⁵S-methionine for 30 min, a variety of newly synthesized proteins were found in the immunoprecipitates (Fig. 1c). Upon chase, these ligands, including p195, p58 and p41, were dissociated from calmegin at various rates. Similarly, the transient interaction of calnexin with nascent proteins was also observed (Fig. 1c). Castanospermine (CAS) treatment of the cells inhibited the association in either case (Fig. 1d), suggesting that the glucosidase activity is needed for the binding process, as shown for calnexin^{3,4}. It should be noted that both chaperones seemed to interact with distinctive ligands, although they may have some ligands in common. We therefore suggest that both calmegin and calnexin interact transiently with nascent glycoproteins during spermatogenesis.

To address the physiological role of calmegin *in vivo*, we generated calmegin-deficient mice by homologous recombination. A

calmegin-targeting construct was designed (Fig. 2a) to interrupt the second exon with a neomycin-resistance gene (*neo*). This insertion disrupted the ATG translation start site. Both the targeting event in D3 embryonic stem cells and the germline transmission of targeted genes were confirmed by Southern blot analysis (Fig. 2b). Intercrosses between heterozygous F_1 mice yielded offspring (24 litters) that segregated in a mendelian distribution: 57 wild type, 104 heterozygous, and 53 homozygous mutant weaning pups. Homozygous ($-/-$) mutant mice did not show any overt developmental abnormalities.

Testes derived from adult F_2 male mice were subjected to analysis of the effect of the *neo*-cassette insertion on calmegin synthesis. The level of full-length (2.4 kb) messenger RNA is reduced to approximately half that of normal in the heterozygous mice (Fig. 2c), but no mRNA is detected in the homozygous mutant mice. When the blots were rehybridized to a probe specific for the calnexin or β -actin gene, constant expressions of both were detected in all F_2 samples. We performed immunoblot experiments to determine whether calmegin protein was present in the mutant mice. The anticalmegin monoclonal antibody (TRA 369) recognized a protein of relative molecular mass 93,000 (M_r 93K) in $+/+$ and $+/-$ males (Fig. 2d). Neither normal-sized calmegin protein nor any smaller fragment was detected in testes from $-/-$ mice. Expression of calnexin in the testis was not significantly affected by calmegin disruption (Fig. 2c,d).

The effect of calmegin disruption on spermatogenesis was analysed histochemically. Adult testis from a $-/-$ mouse was fixed in Bouin's solution and thin sections were made; testicular sections were stained with haematoxylin and eosin or by periodic acid-Schiff (PAS) staining and observed under a microscope. Spermatogenesis was normal in the calmegin $-/-$ tubules, as indicated by the production of mature spermatozoa and the appropriate proportion of cells in each substage. There was no difference in the size of the mutant testis or in the number of germ cells per section of a tubule counted separately according to the meiotic stage (50 ± 8 $+/+$ and 54 ± 9 $-/-$ primary spermatocytes; 144 ± 16 $+/+$ and 144 ± 22 $-/-$ round spermatids; 133 ± 19 $+/+$ and 137 ± 28 $-/-$ elongated spermatids in stages I-IV tubule¹⁰. Three mice from each strain were used for the counting.)

Homozygous mutant males were nearly sterile, but copulation and vaginal plug formation were normal. Even though 11 $-/-$



Figure 4 Sperm from $+/+$ mice successfully adhered to the eggs (**a**), but those from $-/-$ mice failed to attach despite frequent collisions with the zona pellucida (**b**). Original magnification, $\times 400$.

males were mated with 3 females for more than 3 months (more than 120 plugs were observed), only one pregnancy (2 pups) was discovered. There were no significant differences in the fecundity between $+/+$ and $+/-$ males; the average litter size in the wild-type female/heterozygote male cross and the wild-type female/wild-type male cross were (8.2 ± 1.5 , $n = 18$) and (8.4 ± 1.1 , $n = 20$), respectively. In contrast, all F_2 females ($+/+$, $+/-$ and $-/-$) were fertile and showed normal fecundity (data not shown), consistent with our findings that calmegin is not expressed in the ovary^{7,8}.

We collected sperm from the reproductive tracts of females that had been mated with $-/-$ males. The recovered sperm were similar in number and viability to sperm from wild-type males. The motility of sperm from both $+/+$ and $-/-$ mice was maintained at over 80% during the 120 min of incubation in TYH medium. Moreover, motile sperm from $-/-$ mice were present in the oviduct. Epididymal sperm from $-/-$ mice showed normal viability, morphology and motility under microscopic observation. When sperm from calmegin $-/-$ and $+/+$ mice were stained using various polyclonal and monoclonal anti-sperm antibodies, no differences in staining pattern or strength measured by flow-cytometry were found between them (Fig. 3a,b). The biotinylation and subsequent SDS-PAGE analysis of solubilized sperm membrane using avidin-peroxidase also showed no significant difference in sperm membrane from $+/+$ and $-/-$ mice (Fig. 3c). Despite their normal appearance in the *in vivo* fertilization medium, sperm from $-/-$ males were generally unable to penetrate the egg extracellular matrix (Table 1). Removal of cumulus cells showed that these sperm failed to adhere to the egg despite frequent collisions with the zona pellucida (Fig. 4). These results suggest that sperm from $-/-$ males may encounter eggs *in vivo*, but fail to fertilize because they cannot bind to zona pellucida.

Various proteins have been proposed as candidate zona pellucida adhesion molecules on sperm, including sp56 (refs 11, 12), PH-20 (ref. 13), zonadhesin (refs 14, 15), Gal-Tase (refs 16, 17), p95 (refs 18, 19) and sp17 (refs 20, 21). Activity of one or more of these molecules is presumably important in adhesion of sperm to the zona pellucida. Because calmegin transiently interacts with nascent proteins (Fig. 1c) and its gene disruption seems to impair specifically the sperm binding to the egg (Table 1, Fig. 4), we suggest that calmegin may be expressed during spermatogenesis to ensure the proper maturation of certain sperm surface protein(s) required for the egg binding. The identification of protein(s) causing infertility of calmegin knockout mice will provide important insight into the

mechanism of the interaction between sperm and egg. Indeed, it has been reported that about one-third of men with unexplained infertility (semen parameters were within the normal ranges) suffered from no or low binding of sperm to the zona pellucida^{22,23}, although the causes of this condition have remained unknown. The calmegin mutant mice may therefore also provide a model for studying unexplained male infertility. □

Methods

Immunofluorescence staining and flow-cytometry. Frozen sections of adult mouse testis were immunostained with antibodies (TRA369 for calmegin⁸ and alpha-CN IP for calnexin³) as described previously. Sperm were collected from epididymis of calmegin-disrupted and wild-type mice. After fixation with 4% paraformaldehyde, sperm were air-dried on a slide glass and immunostained. The monoclonal antibodies used were OBF13 (ref. 24), MC101 (ref. 25), MN7 (ref. 26), MN9 (ref. 27) and 7C5 (ref. 12) (anti-sp56; QED Bioscience). P. Primakoff provided the polyclonal rabbit anti-PH-20 antibody. For the second antibodies, FITC-labelled anti-mouse and anti-rabbit antibodies (DAKO-immunoglobulins, Denmark) were used. For flow-cytometric analysis, sperm were preincubated in TYH medium²⁸ for 3 h and fixed with 4% paraformaldehyde before staining.

Biosynthetic labelling, immunoprecipitation and biotinylation. Cells isolated from testis were preincubated for 30 min at 33 °C with or without 1 mM CAS in Dulbecco's minimum essential medium deficient in methionine. The cells ($\sim 10^6$ cells) were subsequently labelled for 30 min with 100 μ Ci of [³⁵S]methionine with or without 1 mM CAS and chased. The cells were collected by a centrifugation at 500 g for 5 min and lysed in 150 μ l of 1% Triton X-100/100 mM KCl/10 mM Tris-Cl, pH 7.5, leupeptin (10 μ g ml⁻¹, pepstatin (10 μ g ml⁻¹). The lysates were precleared by incubating with 10 μ l of 20% Zysorb (Zymed) for 20 min on ice followed by centrifugation for 5 min at 10,000 g. Immunoprecipitates were recovered by 1,000 g centrifugation for 3 min after incubating the lysates with anti-calmegin or anti-calnexin antibodies for 1 h on ice followed by incubation with 10 μ l Zysorb for 20 min. After a wash in 0.6 M KCl, 0.05% Triton X-100, 10 mM Tris-HCl, pH 7.5, the immunoprecipitates were resolved on SDS-PAGE and labelled bands were visualized by BAS2000 phosphorimager (Fujix) equipped with a pictography. For biotinylation, live sperm were isolated from epididymis. The biotinylation of sperm surface proteins were performed using ECL protein biotinylation module (Amersham).

Production of calmegin-deficient mice. A clone containing the 5' end of the calmegin gene was isolated from a 129 genomic library. A 7.5-kb *Bam*HI-*Xba*I fragment was subcloned into pBluescript SKII+. The *Sal*I and *Xho*I sites were introduced into the second exon using a Transformer site-directed mutagenesis

kit (Clontech) with mutagenic primer; 5'-AGAGTCGACATGCGTCTC-GAGGGTGTGGA-3'. A *XhoI*-*Sall* fragment of the neomycin-resistance (*neo*) gene from pMC1neo PolyA (Stratagene) was inserted into the *Sall*-*XhoI* sites in the same transcriptional orientation as the calnegin. A *KpnI*-*NotI* fragment was introduced into the pPNT²⁹ vector including the thymidine kinase (*tk*) gene to produce the targeting construct. The plasmid was linearized by *NotI* digestion before electroporation into D3 ES cells. Of 96 G418-gancyclovir-resistant clones, 20 were found to have undergone homologous recombination by Southern blot analysis. Four targeted cell lines were injected into C57BL/6J blastocysts, resulting in the birth of male chimaeric mice. One chimaera produced heterozygous F₁ offspring when bred to either C57BL/6J or 129 females.

In vivo fertilization. Females were superovulated by injection of 5 IU of pregnant mare serum gonadotropin followed 48 h later by 5 IU of human chorionic gonadotropin (HCG). Ovulated egg masses were collected from the oviducts 16 h after HCG injection. Eggs were placed in 200 μ l TYH medium²⁸ drop covered with paraffin oil. Male mice three months old were subjected to *in vitro* fertilization test. Sperm collected as a clot from mechanically dissected caudae epididymidis were introduced into a 200- μ l drop of TYH medium. After 1 h of incubation, about 5 μ l of sperm suspension was added to the drop containing eggs at final concentration of $1-2 \times 10^5$ sperm ml⁻¹. Viability, morphology and motility were observed at various times of incubation. After 1 and 8 h of incubation, eggs were fixed in 4% paraformaldehyde and fertilization events were observed under a microscope. To assess the zona pellucida binding ability, cumulus cells were dispersed by 3-5 min of incubation with hyaluronidase (1 mg ml⁻¹, Sigma type VI-s) and soybean trypsin inhibitor (10 μ g ml⁻¹, Sigma T9003) before insemination. After 20 min of incubation, eggs were fixed and observed.

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Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation

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Chemokines are small secreted proteins that stimulate the directional migration of leukocytes and mediate inflammation¹⁻⁴. During screening of a murine choroid plexus complementary DNA library, we identified a new chemokine, designated neurotactin. Unlike other chemokines, neurotactin has a unique cysteine pattern, Cys-X-X-X-Cys, and is predicted to be a type 1 membrane protein. Full-length recombinant neurotactin is localized on the surface of transfected 293 cells. Recombinant neurotactin containing the chemokine domain is chemotactic for neutrophils both *in vitro* and *in vivo*. Neurotactin messenger RNA is predominantly expressed in normal murine brain and its protein expression in activated brain microglia is upregulated in mice with experimental autoimmune encephalomyelitis, as well as in mice treated with lipopolysaccharide. Distinct from all other chemokine genes, the neurotactin gene is localized to human chromosome 16q. Consequently we propose that neurotactin represents a new δ -chemokine family and that it may play a role in brain inflammation processes.

Three classes of chemokines have been categorized to date: α (CXC), β (CC) and γ (C). The definition for the subclass of chemokines is based on the distance between the first two of four characteristic disulphide-forming cysteine residues. The α -chemokines, including interleukin-8 (IL-8), neutrophil-activating protein 2 (NAP-2), GRO- α - γ , epithelial-cell-derived neutrophil activating protein (ENA-78) and granulocyte chemotactic protein 2 (GCP-2), are mainly chemotactic for neutrophils. By contrast, the β - or CC chemokines are chemotactic for monocytes and, in some instances, for eosinophils or basophils, but not for neutrophils. β -Chemokines include monocyte chemotactic protein (MCP) 1-5, the macrophage inflammatory proteins MIP-1 α and MIP-1 β , eotaxin and RANTES. Lymphotactin is the only γ -chemokine that has only one pair of cysteines and is thought to be chemotactic for lymphocytes⁵⁻⁷. Here we report the discovery of a fourth class of chemokine with significant structural and functional differences to other chemokines.

A cDNA library constructed from murine choroid plexus⁸ was studied by automated high-throughput single-pass sequencing and computer analysis. The predicted amino-acid sequence of one cDNA clone, pT1, exhibited similarity to murine MCP-1. The complete sequence of pT1 was determined and the open reading frame was continuous for 395 amino acids. A full-length cDNA encoding the human homologue of pT1 was further identified, and the predicted open reading frame of this clone (397 amino acids) was 67% identical to that of murine pT1 over its entire length, including a predicted signal peptide of 22 amino acids (Fig. 1). An alignment of the chemokine-like domains from the human homologue of the pT1 gene product (termed neurotactin) with human

IL-8, GRO- α , MCP-1, RANTES and lymphotactin is shown in Fig. 2a. Neurotactin contained the four disulphide-forming cysteines present in all α - and β -chemokines. Both murine and human neurotactin were most closely related to MCP-1 from the corresponding species (40% identical at amino acid level).

The primary structure of neurotactin is notably different from all other chemokines (Fig. 2b). First, it differs in the arrangement of the first two cysteines, by the inclusion of a three amino-acid spacer: CXXXC. Second, the neurotactin precursors are substantially larger than other chemokine precursors, which are usually less than 150 amino acids long. The region of homology to other chemokines is located at the amino terminus approximately from residues 23 to

h-NTN	MAPISLWLLRLATFCHLTLLVLAGQHHGVTKCNITCSKMTSKIPVALLIH	50
m-NTN	MAPSPLAWLLRLAAPPFHLCTLLPGQHLGTMCKEIMCDKMTSRIPVALLIR	50
	*** *	
h-NTN	YQONQASCGKRAIILETRQHLRFCDPKEQWVKDAMQHLDRQAAALTRNG	100
m-NTN	YQLNQESCGKRAIVLETTQHRRCADPKEKWWQDAMKHLDRQAAALTKNG	100
	** *	
h-NTN	GTFEKQIGEVKPRTPPAAGMDSESVVLEPE. ATGESSSLEPTPSSQEAQR	149
m-NTN	GKFEKRVNDVTPGITLATRGLSPSALTKEPESATLEDLALLETTSQEAQR	150
	* *	
h-NTN	ALGTSPELPTGVGSSGTRLPPTPKAQDGG... PVGTGLFRVPPVSTAA	195
m-NTN	TMGTSQEPAAVAVTGSS... LSTSEAQDAGLTAKPQSIGSFEEADIST.T	195
	*** *	
h-NTN	TWQSSAPHQPGPSLWAEAKTSEAPSTQDPSTQASTASSAPEENAPSEGO	245
m-NTN	VWPSPAVYQSGSSWAEAKATESPSTAPSPQVSTTSPSTPEENVGSEGO	245
	** *	
h-NTN	RVMGQGSPPRENSLEREEMGFVPAHTDAFQDWGPGSMHVSVPVPSSEG	295
m-NTN	PPWVQGGDLSPKSLGSEENP... HTDNFQERGPNTVHPVAPISSEE	293
	* *	
h-NTN	TPSREPASGSWTPKAEPIHATMDPQRLGVLTTPVPDAQAATRRQAVGL	345
m-NTN	TPSPELVASGSQAPKIEPIHATADPQKLSVLITPVPDTQAATRRQAVGL	343
	*** *	
h-NTN	LAFLGLLFLCLGVAMFTYQSLQGCPRKMAGEMAEGRLYIPRSCGSNSYVLV	395
m-NTN	LAFLGLLFLCLGVAMFYQSLQGCPRKMAGEMVEGLRYVPRSCGSNSYVLV	393
	***** *	
h-NTN	PV	397
m-NTN	PV	395
	**	

Figure 1 Amino-acid sequence alignment of human and murine neurotactin (abbreviated as NTN). The putative signal peptide is represented by a double underline and the transmembrane domain is underlined. The alignment was generated with the BESTFIT program from the GCG package (Genetics Computing Group, Madison, WI). Identical residues are indicated by asterisks underneath.

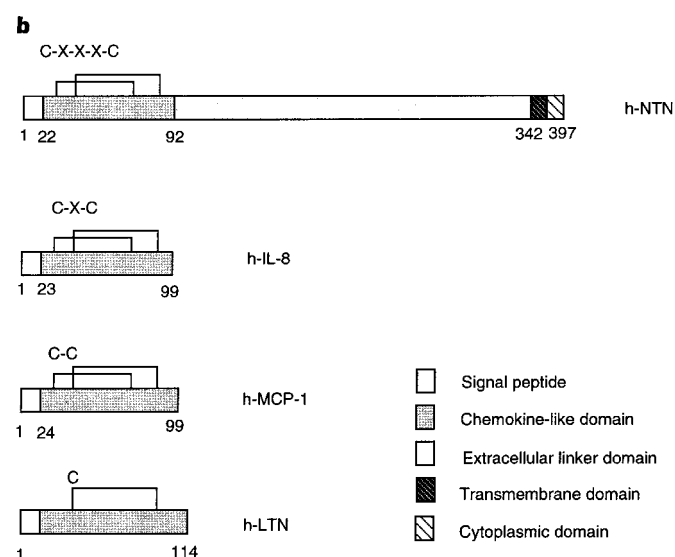
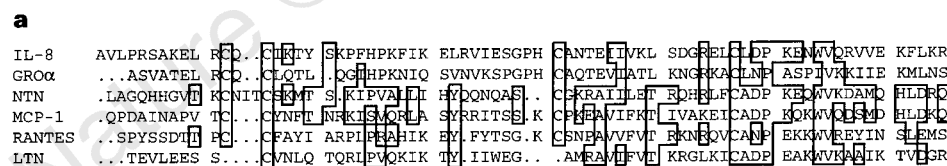


Figure 2 a, Sequence alignment of human neurotactin with α -chemokines IL-8 and GRO- α ; β -chemokines MCP-1 and RANTES and γ -chemokine lymphotactin. Residues identical to neurotactin are boxed. NTN, neurotactin; LTN, lymphotactin. The alignment was generated with the Pileup program from the GCG package with manual modification. **b**, Primary structure comparison of human neurotactin with other subclasses of human chemokines. The representatives for the CC, CXC and C chemokine subfamilies are IL-8, MCP-1 and lymphotactin, respectively. Cysteines that are involved in confirmed (IL-8, MCP-1, NTN) or putative (LTN) disulphide-bond formation are linked by straight lines.

92. Third, neurotactin contains a stretch of 19 conserved hydrophobic residues towards the carboxy terminus, suggesting that it is a membrane protein. Three additional cysteines are present at the C-terminal portion of the neurotactin, one in the transmembrane domain and two in the cytoplasmic domain.

The expression profile of pT1 was determined by RNA (northern) blot analysis of murine and human tissues. Expression of a 3.5-kilobase (kb) mRNA was detected predominantly in murine brain and to a lesser extent in kidney, heart and lung (Fig. 3), as well as uterus (data not shown). Three minor mRNA species were also observed in the brain. This pattern of expression is unusual in the chemokine family. Human neurotactin gene expression was most abundant in the brain and heart, but was also present in all other tissues tested except peripheral blood leukocytes (data not shown). Murine neurotactin mRNA was upregulated in cells of endothelial and fibroblastic origin (data not shown). In EOMA (endothelial cells) and STO (embryonic fibroblasts) the expression of neurotactin mRNA was constitutive and inducible by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and lipopolysaccharide (LPS), whereas in BMS-12 (bone marrow stromal) cells the neurotactin mRNA was detected only after stimulation with TPA or LPS. Neurotactin mRNA was not detected in several cell lines of haematopoietic origin with or without stimulation by TPA and LPS. The tested cell lines were WEHI-3 and Pu5-1.8 (myelomonocytic), P388D1 and

IC-21 (monocytic/macrophage), AKR.G.2 (thymoma), BaF3 (pro B cell), EL-4 (lymphoma), NFS-1.0 (B-cell lymphoma) and BCL (B-cell leukaemia).

In order to determine the association of neurotactin with the cell membrane, a construct containing the full-length neurotactin coding region was made in mammalian expression vector pN8e vector (kindly provided by J. Morgenstern and K. Thieriault). A FLAG epitope tag was added to the C terminus. As shown in Fig. 4, strong staining was detected on the surface of 293 EBNA cells transiently transfected with this construct, suggesting that full-length neurotactin is indeed membrane-anchored. The signal was competed by FLAG peptide.

Two soluble versions of murine neurotactin, corresponding to the chemokine-like domain (P1, from residue 22 to 105) and entire extracellular domain (P3, from residue 22 to 337) were constructed and expressed as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli*. Purified P1 was analysed by matrix-assisted laser desorption ionization mass spectrometry after a *Staphylococcus aureus* V8 protease digestion to examine its disulphide formation (data not shown). Under non-reduced conditions two proteolytic fragments were recovered which represent the covalent attachment of peptides (F-1)-E33 to S57-R66 and I34-E56 to T67-E79, respectively. These peptides encompass the C32-C58 and C36-C74 disulphide pairings, respectively. Under reducing conditions in the presence of

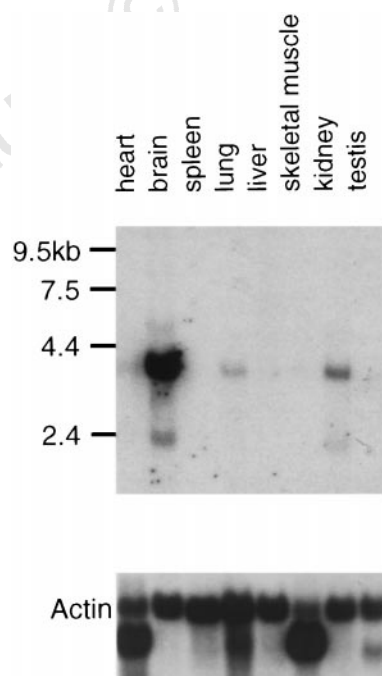


Figure 3 Murine tissue northern blot probed with a full-length murine neurotactin cDNA probe. Hybridization of the same blot with actin probe is shown at the bottom to demonstrate equal loading RNA in each lane.

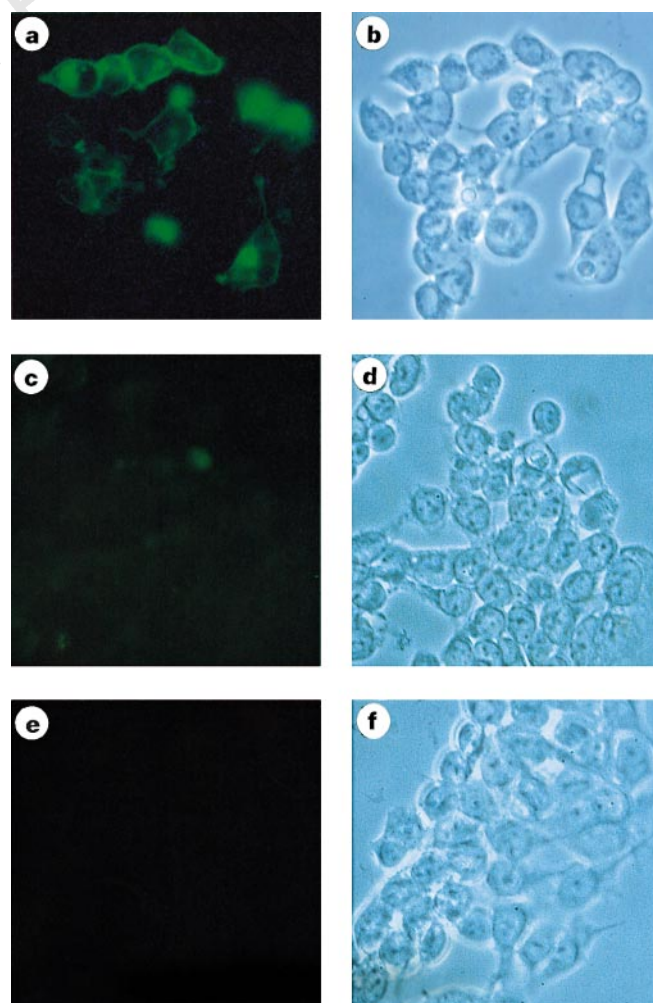


Figure 4 Immunofluorescent labelling of transfected 293 cells. **a, b**, Fluorescent staining and light field image from transfected 293 cells. **c, d**, Fluorescent staining and light field image from transfected 293 cells after competition with FLAG peptide. **e, f**, Fluorescent staining and light field image in mock transfection.

DTT, the consistent peptides of the pairs were observed along with complete absence of the disulphide-bonded peptides substantiating the assignment. No additional ions were detected that would support alternative disulphide pairing and thus indicate proper folding of the domain with respect to homologous chemokines.

Purified P1 and P3 were subsequently assayed for their chemotactic specificity *in vitro*. As shown in Fig. 5, recombinant neurotactin P1 was found to be chemotactic for human neutrophils *in vitro* with a maximal activity at 10^{-8} M ($n = 3$), whereas P3 has no significant effect on all three types of human leukocyte tested. The same chemotaxis index for IL-8 has been documented⁹. P1 was also chemotactic for human T lymphocytes. Further testing showed that neither P1 nor P3 had chemotactic effect on human monocytes, human myelomonocytic THP1 cells and murine P388D1 monocytic cells (data not shown). P1 and P3 were subsequently injected into mice peritoneally to determine their chemotactic efficacy *in vivo*. Bacterial lysate containing only GST was purified in the same fashion as P1 and P3, to serve as a control for the *in vivo* assay. Two hours after injection, peritoneal exudate was collected and assayed for leukocyte infiltration (Fig. 6). Consistent with the *in vitro* results, P1 was chemotactic for neutrophils and lymphocytes *in vivo*. However, P1 *in vivo* was also chemotactic for monocytes, but not for eosinophils. The activity of P1 towards monocytes is probably due to a secondary effect *in vivo* and therefore may not reflect its chemotactic specificity. The inability of P3 to chemotact monocytes and lymphocytes *in vivo* was also consistent with the *in vitro* data, but in contrast to the observations *in vitro*, P3 was chemotactic for neutrophils, suggesting differential activation of neurotactin *in vivo*.

Neurotactin expression in normal and LPS-treated murine brain was determined by immunohistochemistry. Frozen brain sections from normal, LPS-treated mice were stained with an anti-neurotactin peptide antibody with or without peptide competition (Fig. 7a–f). In normal brain, staining was localized to capillary vessels and resident microglia. Two hours after LPS treatment, increased labelling intensity on the same cell types was observed. An increasing number of the activated microglial cells stained positive for the anti-neurotactin antibody. In both normal and LPS-treated brain, staining of larger vessels was restricted to the apical region of the endothelium. It is known that, in addition to resident microglial cells, two other subtypes of microglial cell are present in the central nervous system at the blood–brain barrier: perivascular (completely surrounded by basal lamina), and juxtavascular microglia (directly in contact with basal lamina)¹⁰. The anti-neurotactin antibody staining associated with micro-vessels was consistent with staining of those microglial cells.

Paraffin-embedded brain sections from mice with severe experimental autoimmune encephalomyelitis (EAE) were examined to evaluate neurotactin expression further. EAE develops spontaneously in immunodeficient α -myelin basic protein T-cell receptor transgenic mice¹¹ or in mice that have been actively immunized with proteolytic peptide (PLP)³⁰, and these have been used as a model of chronic inflammatory diseases. Neurotactin was found to be up-regulated in activated microglia from such EAE mice, in a similar manner to LPS-treated mice (Fig. 7g–h).

Both human and murine neurotactin genes were located on different chromosomes from other chemokines. The genes for the α -chemokines have been mapped to human chromosome 4 and 10 (ref. 31), and the β -chemokines to human chromosome 17 and mouse chromosome 11 (ref. 3). Lymphotactin was located to chromosome 1 in both human and mouse^{5,6}. Using a panel of backcross progeny of C57Bl/6J *Mus musculus* and *Mus spretus* mice the murine neurotactin gene was mapped to the long arm of chromosome 8 (data not shown). The human neurotactin gene was localized by radiation hybrid mapping to the syntenic region on chromosome 16q (data not shown). This result further supports the notion that neurotactin represents a new class of chemokine.

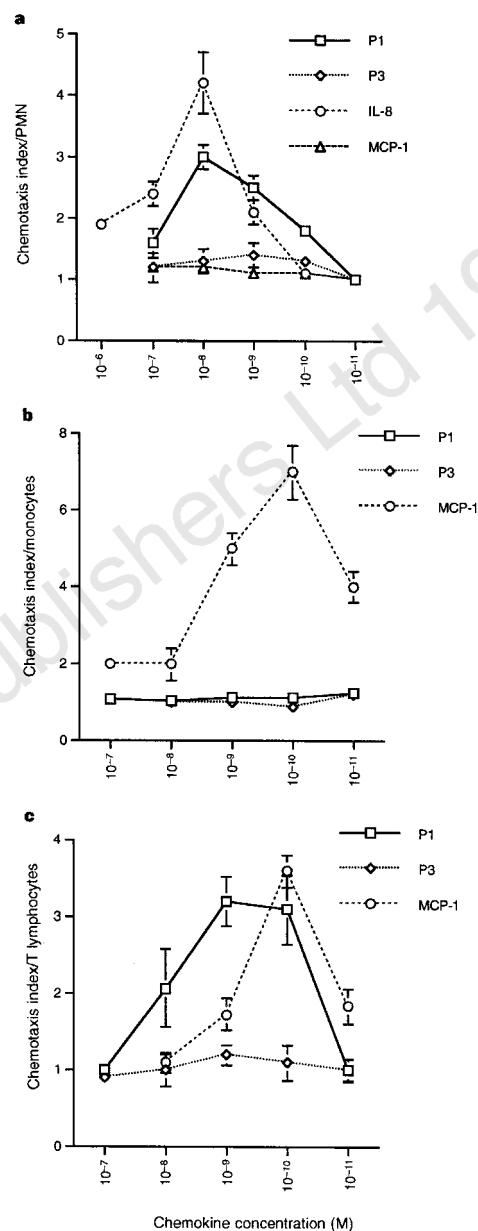


Figure 5 Chemotaxis assay of recombinant murine neurotactin on purified human neutrophils (a), monocytes (b) and lymphocytes (c). The chemotaxis index is calculated as the ratio of the number of leukocytes migrated onto the sample filter to those on the control filter ($n = 3$). The percentage migration of neutrophils and lymphocytes induced by P1 (at the optimal concentrations) is 12% plus or minus 3% and 6.5% plus or minus 0.3%, respectively. The background migration for neutrophils and lymphocytes is 4.5% plus or minus 2% and 2.3% plus or minus 0.5%.

The unusual pattern of cysteine residues (CXXXC) and membrane association suggest that neurotactin represents a new class of chemokine and may have new functions through membrane association that contrast with soluble chemokines. Several chemokines, such as IL-8 and GRO- α , have been shown to act locally because of their association with the extracellular matrix¹², and by doing so to establish a gradient in the blood flow. Although it is unknown whether neurotactin functions through a membrane-bound or soluble form, or both, the membrane association of neurotactin represents a new mechanism for chemokine presentation, localization and juxtacrine signalling. Juxtacrine stimulation is found in a wide range of species including insects and mammals, and is thought to

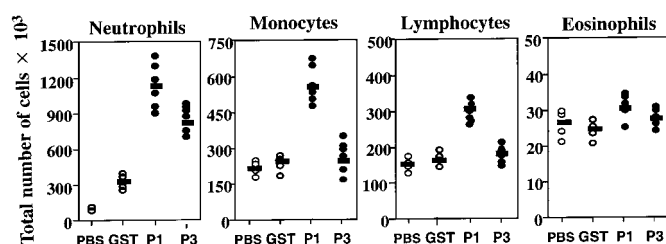


Figure 6 Neurotactin-induced recruitment of leukocytes to the peritoneum. Peritoneal exudate was collected from C57BL/6J mice 2 h after injection of either P1 or P3 neurotactin (filled symbols), PBS or GST (empty symbols). Each dot represents one individual mouse analysed (6 and 4 mice per experimental and control group, respectively). The bar in each panel represents the mean of the total number of cells of the leukocyte type indicated.

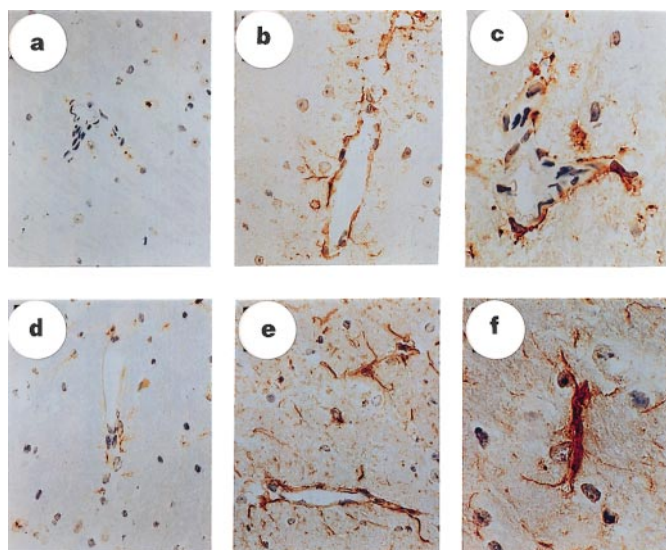


Figure 7 Expression of murine neurotactin in normal, LPS-treated and EAE brain. Normal murine brain sections stained with anti-neurotactin polyclonal antibody, with (a) or without (b) peptide competition (original magnification × 250). c, Higher magnification of an area containing vascular endothelium (original magnification × 1,000). LPS-treated murine brain sections stained with anti-neurotactin polyclonal antibody, with (d) or without (e) peptide competition. f, Higher magnification of a microglial cell. Paraffin sections from control (g) and EAE (h) mice stained with anti-neurotactin polyclonal antibody (original magnification × 250).

be important in developmental processes that have spatial restrictions¹³. There are many examples of other cytokine classes that can be membrane associated or secreted, such as steel factor (SLF), macrophage colony-stimulating factor, epidermal growth factor (EGF) and members of the tumour necrosis factor (TNF) family¹³. Lack of the membrane-associated form of SLF in *sl/sl* mice causes pathology despite the presence of a soluble form^{14,15}.

By sequence comparison, neurotactin is most closely related to MCP-1, although functionally it seems to be different from the β -chemokines based on chemotaxis data with recombinant proteins. Both *in vitro* and *in vivo*, recombinant neurotactin P1, which contains the chemokine domain only is chemotactic for neutrophils, resembling the α -chemokines, such as IL-8. However, from the limited tissues and cells tested, neurotactin gene expression is overlapping but different from IL-8. For example, they are both expressed in fibroblastic and endothelial cells; however, by northern analysis, neurotactin RNA was not detected in nine murine B, T and monocyte/macrophage cell lines or human peripheral blood leukocytes. It is also interesting to note that neurotactin does not contain the 'ELR' motif present in other neutrophil-chemoattractant chemokines^{16–18}.

Our results have demonstrated that the chemokine-like domain (P1) and complete extracellular domain (P3) of neurotactin are differentially active *in vitro* and *in vivo*. Principally the entire extracellular domain construct (P3) is incapable of inducing chemotaxis of leukocytes *in vitro*, yet it is chemotactic for neutrophils *in vivo*. One explanation for the difference is that P3 undergoes further post-translational processing *in vivo* to generate a form more analogous to P1 that is able to chemoattract neutrophils. Our data suggest the possibility that multiple forms of neurotactin exist with different ranges of activity controlled by post-translational proteolysis.

Primary sequence analysis of neurotactin indicates the possibility for the generation of secreted or cleaved forms of neurotactin. Both human and murine neurotactin contain a conserved dibasic poten-

tial protease cleavage site that lies towards the N terminus of the predicted transmembrane domain (R339–340 in human and R337–338 in mouse). Pairs of basic residues have been shown to serve as the signals for precursor cleavage by mammalian proteases such as furin and PC3 (ref. 19) and might also enable the release of a soluble version of neurotactin. The C-terminal residue of both human and murine neurotactins is valine. A C-terminal valine residue in membrane-anchored proTGF- α and steel factor is essential for their extracellular proteolytic cleavage²⁰. Alternatively spliced forms of neurotactin mRNA, such as those observed in the brain (Fig. 3), might also result in shorter soluble forms, although the molecular nature of these have yet to be defined.

The induction of neurotactin by inflammation and the ability of recombinant neurotactin to cause leukocyte chemotaxis suggests a role in inflammatory responses. Neutrophil demargination and infiltration in the brain is characteristic of acute bacterial infections such as sepsis. It also occurs early in cerebral ischaemia, stroke and brain trauma²¹. The increased expression of neurotactin in brain microglia 2 h after LPS administration suggests rapid upregulation following inflammation. Furthermore, the high levels of neurotactin expression in EAE brain by activated microglia indicates that neurotactin might also be involved in the generation and progression of other brain inflammatory diseases, such as multiple sclerosis.

Given the many unique properties of neurotactin we propose that it is the first of a new class of chemokines, and by convention we propose that this new class of chemokines be termed the δ -family of chemokines. □

Methods

High-throughput sequencing and computer analysis. DNA was prepared from individual transformants using the AGTC (Gaithersburg, MD) system in a 96-well format. Samples were sequenced by dye-primer fluorescent chemistry on automated ABI373 and ABI377 machines. Expressed sequence tag (EST) sequences were automatically processed after filtering out *E. coli*, ribosomal or

mitochondrial contaminants. Next, vector and repetitive elements were masked and/or removed from each sequence. Sequences were then searched against the Genbank nucleotide database using the BLASTN program²², and through a non-redundant protein database using the BLASTX program^{22,23}. More detailed analysis of individual DNA sequences was done using the GCG package of programs. Contig assembly of sequences was done using Sequencher (Gene Codes Corp., Ann Arbor, MI). The putative signal peptide was predicted by a signal-peptide prediction program²⁴. The human homologue was initially identified as a partial sequence entry in dbEST. The clone was obtained from Genome Systems Inc. (St Louis, MO) and the complete sequence was determined as described above.

Northern blot analysis. Northern blots were probed using standard techniques²⁵ with a ³²P-labelled DNA fragment encoding the full-length pT1. Northern blots from multiple murine tissues and control actin probe were obtained from Clontech (Palo Alto, CA).

Detection of membrane-associated neurotactin. Full-length murine neurotactin cDNA was modified for expression in mammalian system by polymerase chain reaction (PCR) with the following primers: 5'-GGGAAA-GAATTCATGGCTCCCTCGCCGCTCGGTGG-3' and 5'-GGGAACTCGA-GTCATTATCATCATCACTTTATATCCACTGGCACCAGGACGTATGA-3'. Nucleotides coding for DYKDDDDK (FLAG epitope) were incorporated into the 3' reverse primer for subsequent detection by the M2 anti-FLAG antibody. The PCR products were cloned into the pN8e vector bearing the EBV origin of replication. The construct DNA was prepared with the Qiagen Maxiprep kit (Qiagen, Chatsworthy, CA) and transfected into 293 EBNA cells with lipofectamine (Gibco, Gaithersburg, MD) in 8-well chamber slides. 48 hours after transfection, the cells were fixed with 50% methanol and 50% acetone for 1 min at room temperature. After washing with 2.5 ml Tris-buffered saline four times, the cells were first incubated with 10 µg ml⁻¹ of M2 anti-FLAG monoclonal antibody (Eastman Kodak, New Haven, CT) and then with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody at 1:1,000 dilution (Jackson Immuno Research, West Grove, PA) for 1 h each. The immunofluorescent staining was visualized with a Zeiss Axioskop 20 with ×200 magnification.

Recombinant murine neurotactin expression in *E. coli*. Soluble murine neurotactin was constructed by PCR using modified oligonucleotides. The forward primer for P1 construct was 5'-GGGAAAGAATTCCTGCCGGTGTCAG-CACCTCGGCATG-3' and the reverse primer was 5'-GGGAACTCGAGT-CACCTTCTCAAACCTTGCCACCAATTTT-3'. The reverse primer for the P3 construct was 5'-GGGAACTCGAGTCACCTTGTGGCTGCCTGGGTGTC-GGG-3'. pfu *Taq* polymerase was purchased from Stratagene (La Jolla, CA). The PCR products were ligated to pGEX-4T (Pharmacia, Piscataway, NJ) by *Eco*RI and *Xho*I sites and transformed into *E. coli* DH5α (LifeTechnology, Gaithersburg, MD). Recombinant protein was induced, purified with glutathione-Sepharose and cleaved from GST according to the manufacturer's instructions. The products P1 and P3 contain five additional amino acids (GSPEF) at the N terminus resulting from thrombin digestion of GST fusion proteins. As a control, GST was prepared along with P1 and P3 at the same time. The same amount of thrombin was added to the control GST before binding to an endotoxin BX column. Endotoxin was removed from the preparation using an endotoxin BX column (Cape Cod Associates, Falmouth, MA) and was determined to be <0.01 EU ml⁻¹.

Chemotaxis assay. The ability of neurotactin to elicit chemotaxis was tested in an *in vitro* assay as described for PMN²⁶, monocytes²⁶ and lymphocytes²⁷. Neutrophils, monocytes and T lymphocytes were isolated from human peripheral blood as described²⁷⁻²⁹. Preparations were greater than 90% pure by Wright-Giemsa staining. 50,000 neutrophils were added to each well of a 48-well micro chemotaxis chamber containing a 5-µm pore-size filter. Human IL-8 and MCP-1 were purchased from Biosource International (Camarillo, CA). Varying concentrations of neurotactin and other chemokines were added to the lower chamber of each appropriate well. The chamber was incubated at 37°C, 5% CO₂ for 30 min. Stained and fixed migrated cells were quantified by counting three high-power fields (×400 magnification) per well. The number of cells that migrated to the buffer control were subtracted as background.

The *in vivo* chemotactic migration of leukocytes to neurotactin was determined by injection of either P1 fraction or P3 fraction of neurotactin protein into the peritoneum of C57BL/6J mice. Two hours after neurotactin

(0.5 µg in 400 µl PBS per mouse) or control protein (GST, 0.5 µg in 400 µl PBS per mouse) or PBS administration (400 µl per mouse), peritoneal exudate was collected and the number and subtype of leukocytes recovered in this fluid determined. Number and type of leukocytes was determined for four high-power fields (×40 magnification; total area 0.5 mm²) per section and area. Number of neutrophils and eosinophils were determined by staining with Wright-Giemsa. Macrophages and monocytes were assayed by Moma-2 antibody. Lymphocytes were assessed by Thy 1.2 (53-2.1) and IgM (II/41) immunostaining.

Neurotactin expression in normal, LPS-induced and EAE murine brains by immunohistochemistry. Polyclonal anti-neurotactin antibody was raised in rabbit against peptide LPGQHLMGTMKCEIM at the N terminus of the protein (Research Genetics, Huntsville, AL). Antibody was affinity-purified from 12-week bleeds. Eight-week-old CD1 mice were injected intravenously with 40 µg LPS and then killed by cervical dislocation after 2 h. The brain was excised, bisected transversely before each piece was rolled in Tissue Tek OCT compound (Cryoform, IEC, MA) snap frozen in isopentane/dry ice and stored at -70°C. Sections (3 µm) were cut onto microscope slides, air-dried, fixed in 2% paraformaldehyde (5 min, 4°C) and methanol (10 min, -20°C). Brain from mice suffering active EAE were fixed in 10% neutral buffered formalin before paraffin embedding. Sections (4 µm) were microwaved twice for 5 min in 0.01 M sodium citrate (pH 6) before staining. Fixed sections were stained with antibody using an avidin/biotin staining method. All incubations were done under humidified conditions and slides were washed twice between steps for 5 min each in 0.1 M PBS supplemented with 0.2% gelatin (PBSG). Sections were overlaid with 20% fetal calf serum in PBS for 15 min and then incubated overnight at 4°C with polyclonal anti-neurotactin or normal rabbit serum (both diluted to 1/200 in PBS supplemented with 0.1% BSA). Endogenous peroxidase was blocked by incubation for 20 min in methanol containing 0.3% hydrogen peroxide. Nonspecific staining due to cross-reaction with endogenous avidin or biotin was blocked by incubation with avidin solution followed by biotin solution, both for 20 min. Bound monoclonal antibody was visualized by incubation with biotinylated swine anti-rabbit immunoglobulin (Dako, CA) and then streptavidin peroxidase complex both diluted in 10% normal mouse serum PBS, and incubated for 1 h. Finally, slides were flooded with peroxidase substrate solution (400 µg diaminobenzidine in 10 ml PBS, containing 0.01% hydrogen peroxide) for 10 min before counter-staining with haematoxylin. Control sections were included where monoclonal antibody, biotinylated anti-rabbit immunoglobulin or streptavidin complex were selectively omitted. In addition competitive inhibition of the antibody was accomplished by pre-incubation of antibody with the peptide (25 µg ml⁻¹) for 45 min at 37°C before incubation with tissue sections.

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Ligand-specific oligomerization of T-cell receptor molecules

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T cells initiate many immune responses through the interaction of their T-cell antigen receptors (TCR) with antigenic peptides bound to major histocompatibility complex (MHC) molecules. This interaction sends a biochemical signal into the T cell by a mechanism that is not clearly understood. We have used quasi-elastic light scattering (QELS) to show that, in the presence of MHC molecules bound to a full agonist peptide, TCR/peptide–MHC complexes oligomerize in solution to form supramolecular structures at concentrations near the dissociation constant of the binding reaction. The size of the oligomers is concentration dependent and is calculated to contain two to six ternary complexes for the concentrations tested here. This effect is specific as neither molecule forms oligomers by itself, nor were oligomers observed unless the correct peptide was bound to the MHC. These results provide direct evidence for models of T-cell signalling based on the specific assembly of multiple TCR/peptide–MHC complexes^{1–4} in which the degree of assembly determines the extent and qualitative nature of the transduced signal⁵. They may also explain how T cells maintain sensitivity to antigens present in only low abundance on the antigen-presenting cell.

The 2B4 TCR recognizes and interacts specifically with the moth

cytochrome *c* (MCC) peptide, presented by the murine class II MHC molecule E^k, with a dissociation constant (K_D) of 40–90 μ M (refs 6, 7) (Table 1). To follow the oligomerization state of the two proteins in solution, the soluble forms of the two molecules were studied using QELS, which allows the rapid determination of the translational diffusion coefficients (D) of particles dispersed in solution. These coefficients can then be used to derive the size of the particles by using the Stokes–Einstein relation (see Methods).

Soluble 2B4 TCR is characterized by a unimodal intensity distribution with an average hydrodynamic radius (R_h) of 3.8 ± 0.04 nm (Fig. 1). Almost identical values are observed for E^k molecules complexed with wild-type MCC peptide (3.7 ± 0.7 ; Fig. 1), as well as with two other MCC variants, 102S (3.6 ± 0.5) and 99A (3.7 ± 0.5) (data not shown). This similarity in R_h between 2B4 and E^k is expected as the soluble form of both molecules has virtually the same apparent relative molecular mass (70K) on SDS–PAGE gels, and both structures can be accommodated into oblate ellipsoids of similar dimensions. Gel-filtration experiments further demonstrate this point, showing that the two molecules have essentially identical retention times on a Superdex-200 column (data not shown). To estimate the size expected for a single TCR/peptide–MHC complex, we also constructed a dimeric form of E^k by using an MCC peptide dimerized at its free amino terminus with a disulphide bond. QELS analysis on this dimer preparation gave an R_h of 5.5 nm (Fig. 1).

The results observed for 2B4/MCC–E^k mixtures at different protein concentrations are shown in Fig. 2. At concentrations well below K_D , the intensity distribution profile peaks at 3.8 nm, which is virtually the same as for the monomeric species (Fig. 2a). Thus, in accordance with the kinetic data (Table 1), interaction between the two molecules is minimal at this concentration. At a higher concentration, a clear shift with a maximum centred at 5.5 nm is observed. This value is consistent with a single TCR/MCC–E^k ternary complex, as suggested from results obtained for the peptide-linked E^k dimers described above. It is also in agreement with calculations ($R_{h,calc} \approx 5.2$ nm) based on the R_h values determined for the two molecules, after correcting for anisotropy and hydrodynamic interactions effects (Fig. 2 legend). Analysis of the change in the scattered intensity of the mixture as compared to the monomer

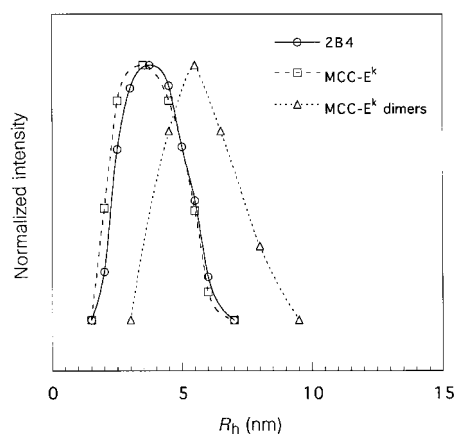


Figure 1 Distribution of scattered intensity versus R_h as obtained for 2B4, MCC–E^k and MCC–E^k dimers. The intensity data, like those in Fig. 2, are presented on an arbitrary scale in which all values are normalized to that of the highest peak. True peak widths are generally broadened by calculation of the intensity distribution through the regularization procedure as used here. Protein concentrations were 21.4 (2B4 and MCC–E^k) and 10.7 μ M (E^k–MCC dimers). For the preparation of MCC–E^k dimers, two E^k molecules were connected through a dimeric MCC peptide containing a flexible linker. In this and the following figures, results shown are representative or the average of 2–3 measurements; uncertainty involved in measurements was generally ≤ 0.5 nm.

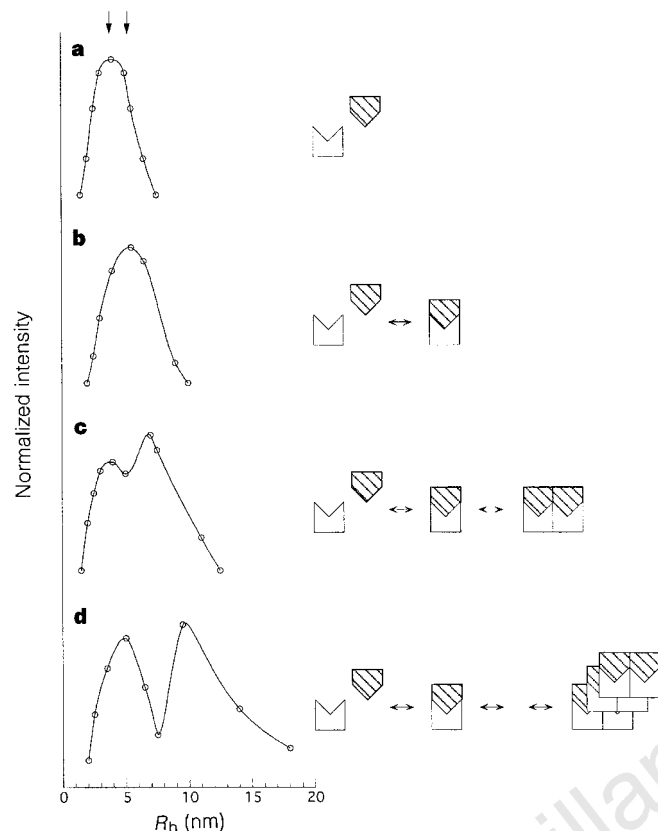


Figure 2 Oligomerization of 2B4/MCC- E^k . Total protein concentration was 11.4 (a), 85.7 (b), 100 (c) and 114.3 μM (d). In this and all other mixing experiments, the molar ratio between the two macromolecules was 1 and, before mixing, the two molecules were measured separately to ensure their monomeric state. The two arrows shown indicate the positions determined experimentally for the monomeric species of the molecules and for the E^k -MCC dimers. R_h of the latter is expected to deviate from that calculated for the TCR/MHC complex by no more than 9–10%, if a side-by-side orientation is assumed². Less acute conformations (that might be induced by the peptide linker) and 2B4 glycosylation will both act to decrease this difference. Model calculations were made using the Kirkwood formalism²³ (and see also ref. 24), or by using a simplified method in which structures were approximated as ellipsoids of revolution and their translational frictional coefficients (f) (which relate to the translational diffusion coefficient by: $D = kT/f$), were corrected for anisotropy effects by using axial ratio-based correction (Perrin) factors. For the simple structures shown in the text, the two methods have yielded similar results. Models shown to the right of the spectra are simplified. In reality, oligomers of smaller or larger sizes than the ones shown are likely to exist.

sample led us to estimate about 35% binding at this concentration, consistent with 31% expected for a reaction with a K_D of 65 μM .

At higher concentrations, the formation of higher-order structures, composed of TCR/peptide-MHC assemblies, becomes evident. At a total protein concentration of 100 μM , the mixture gives rise to an intensity distribution in which two maxima are apparent (Fig. 2c): the first of these correlates with the peak exhibited by the monomeric forms of the molecules; the second, however, is significantly shifted to the right and is centred at 7 nm, 1.5 nm larger than that of the ternary complex. This probably corresponds to a TCR/peptide-MHC 'superdimer', (TCR/peptide-MHC)₂, for which a value of 6.5 nm was calculated, based on previously proposed models^{2,8}. Unlike some hormone and other cell-surface receptors, for which ligand binding is usually followed by a single dimerization step (for a review, see ref. 9), the effect reported here is

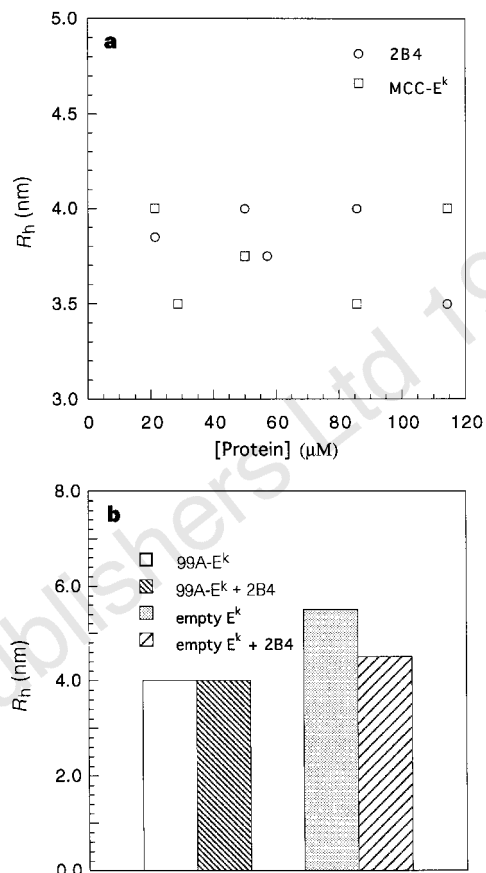


Figure 3 Complex formation is necessary for TCR/MHC oligomerization. **a**, Titration of isolated 2B4 and MCC- E^k . **b**, Mixing experiments involving 2B4 and null E^k complexes. Total protein concentration was 114.3 μM .

titratable. When protein concentration is increased further (to $\sim 114 \mu\text{M}$; Fig. 2d), the second peak is shifted even further to the right ($R_h = 9.5 \text{ nm}$), allowing better resolution of the two peaks. The first peak is also shifted, though only moderately, suggesting that equilibrium between the different oligomeric species is now driven to the right of the kinetic schemes provided in Fig. 2. Moving beyond a simple superdimer arrangement, an R_h of 9.5 nm could be accounted for by several oligomerization schemes, depending on the molecular configuration of the aggregates. Assuming, however, that the ternary complexes are roughly on the same plane, as shown in Fig. 2d, it would take approximately six TCR/peptide-MHC complexes to give this R_h value. This configuration would also maximize the buried surface area between its adjacent superdimers. Finally, the changes seen in the oligomeric state of the molecules are reversible; diluting a concentrated sample to approximately 14 μM

Table 1 Kinetic parameters for 2B4 TCR binding to E^k complexes

E ^k complex	<i>k</i> _{on} (M ⁻¹ s ⁻¹)	<i>k</i> _{off} (s ⁻¹)	<i>K</i> _D (μM)	T-cell activation
MCC	600†–1,600‡	0.057†–0.063‡	50–90	agonist (ref. 7)
99A	n.d.	n.d.	>2,000	none (ref. 7)
empty E ^k	n.d.	n.d.	>2,000	none (ref. 22)

Kinetic parameters were determined from surface plasmon resonance measurements. Rate constants shown for MCC–E^k binding to 2B4 were derived using either amine⁶ or cysteine⁷ coupling chemistry. Data shown for 99A–E^k and empty E^k were derived from both direct binding and solution inhibition experiments. n.d., Not determined.

resulted in a decrease in *R*_h back to its original monomeric values (data not shown).

The specificity of oligomerization is clear from several experiments, including one in which individual 2B4 and MCC–E^k preparations showed no change in their oligomeric state when analysed at concentrations up to ~114 μM (Fig. 3a). In addition, 2B4 was also incubated with two different E^k preparations not recognized by this TCR (Table 1), including E^k complexed with the K99A variant of MCC. As shown in Fig. 3b, no changes in *R*_h could be observed for this complex on mixing with 2B4. The second null variant tested was E^k without a bound peptide. In the concentration range used for the analysis, E^k on its own exists as a dimer (Fig. 3b). When 2B4 is added, however, the *R*_h observed is 1.0 nm lower than that determined for E^k alone. This reduction in *R*_h is due to the contribution of the monomeric 2B4 (which has a significantly smaller *R*_h) to the intensity distribution profile, and indicates that the two species together are virtually inert. Thus oligomerization does not occur in this system unless preceded by a specific binding step between the TCR and the MHC.

Numerous experiments have suggested that the multimerization of T-cell receptors is important in T-cell activation^{1–4} and development⁴, but there has been no direct evidence for such entities or information about their composition. Here we show that, following engagement, TCR/peptide–MHC complexes oligomerize spontaneously in solution to form supramolecular structures. This process is specific, titratable and occurs at concentrations near the *K*_D of the primary binding reaction. Clustering of TCR molecules at the cell–cell contact plane¹⁰ and the reduction in the degree of freedom of these molecules at the cell surface are likely further to amplify this effect. Although TCR/peptide–MHC conjugates oligomerized without any other molecules being present, this process might be further enhanced by interactions with auxiliary co-receptors, such as CD4 or CD8. In this context, it was recently shown that the interaction between the TCR and its specific MHC–peptide complex was strengthened significantly in the presence of homo- or heterodimeric CD8 molecules¹¹. Moreover, this effect of CD8 could be exerted in a substoichiometric fashion, potentially through a rapid serial engagement. Thus CD4 and CD8 molecules may interact rapidly with a series of TCR/peptide–MHC complexes, leaving behind long-lived conjugates which might then have a higher probability to oligomerize.

The structural basis underlying the oligomerization of 2B4/MCC–E^k is unknown. One possible explanation is that low-affinity homotypic interactions in either of the macromolecules are stabilized on ligand binding, promoting subsequent oligomerization. Homodimers, though of differing geometry, were observed in crystals of class II DR (refs 2, 12) and E^k (ref. 13) molecules, as well as those of Vα TCR⁸, but are absent in heterodimeric αβ TCR structures¹⁴. Alternatively, small but significant conformational changes might be induced in one or both of the proteins on binding, thus enhancing complementarity between adjacent complexes. In this regard, structural analysis of a TCR/peptide–class I MHC complex¹⁵ revealed small changes in conformation that correlate with ligand binding in the cognate MHC, but their relevance to the alternative described above is not clear.

The titratable oligomerization observed here may explain how slightly different antigens could produce large differences in the

extent and character of a T-cell response. In such a case, the amount and degree of TCR/peptide–MHC aggregation that occurs after binding is strongly dependent on the stability of the primary complex. As such, a requirement for oligomer formation is akin to kinetic proof-reading⁵ or discrimination¹⁶ models. These models propose that small differences in binding energy can be amplified if the primary and the final (activating) complexes are temporally separated by one or several intermediates. Indeed, an oligomerization-based mechanism has been proposed⁵ in the context of such a model. A distinguishing feature of these models is that they allow, in addition to a simple 'on'/'off' distinction between agonist and null antigens (as demonstrated here), for a continuous gradation of responses, including that of antagonism. A corollary to this model is that small or metastable clusters may generate signals distinct from those exerted by larger or more stable aggregates. Given this, antagonism would be expected for MHC–peptide complexes with a moderate affinity towards the TCR (that is, between those of null and full agonistic peptides), as has been demonstrated^{7,17}. There are two possible origins for this antagonism: first, owing to differential occupancy effects, small, rapidly dissociating clusters might physically interfere with the formation of stable, 'productive' aggregates; second, the small, metastable aggregates might produce 'negative' signals or use up limiting substrates⁷.

Our data also have implications for the problem of how a small number of MHC molecules containing a specific peptide are significantly concentrated at the T-cell interface. Although some enhancement of specific peptide–MHC molecules could be achieved by co-capping with their cognate TCRs on the cell surface, there would still be many MHC molecules containing irrelevant peptides¹⁸. In agreement with previous models^{2,19}, the effect we show here suggests that 'successful' TCR/peptide–MHC complexes have an inherent affinity for each other as opposed to unligated TCRs or MHCs, and as such they can interact preferentially and oligomerize under limiting antigen conditions. □

Methods

Soluble proteins. Soluble glycosyl-phosphatidylinositol (GPI)-linked 2B4 TCR was prepared according to ref. 20, but with the following modifications. Following cleavage from Chinese hamster ovary (CHO) cells expressing GPI-linked 2B4, collected cells were affinity-purified on an A2B4 (anti-Vα) antibody column followed by a H57 (anti-Cβ) purification step. The resulting TCR was then size-purified on a Superdex-200 size exclusion column (Pharmacia). Soluble GPI-linked E^k was prepared and purified⁶. Peptide–E^k complexes were prepared by incubation of E^k with peptides for 3 days at 37 °C in citrate/phosphate buffer (pH 5.1), using a 30-fold molar excess of peptide. Peptide-loaded E^k was then size-purified as outlined⁶. After purification, more than 85% of the molecules are found to contain a bound peptide. For the preparation of MCC–E^k dimers, MCC peptide (sequence: ANERADLIAYLKQATK) containing a Gly-Ser extension and a C-terminal Cys residue (MCC-SSGGGSGSGGSGC-CPNH₂) was oxidized to a disulphide and purified by reverse-phase (C18) high-pressure liquid chromatography (HPLC; Rainin Instruments). Dimers were produced by first incubating a large excess of dimeric peptide with E^k at pH 5.1, as described above. After 2 days, the peptide-loaded monomeric E^k was isolated by gel-filtration and was further incubated for 3 days at pH 5.1, with a twofold molar excess of empty E^k. Dimeric E^k was size-purified as described and eluted as expected for such a configuration, well separated from the monomeric form. Wild-type MCC, as well as other MCC variants, were synthesized by f-MOC chemistry at Stanford's Protein and Nucleic-Acid (PAN) Facility, and were HPLC-purified on a C18 reverse-phase column.

Surface plasmon resonance. Soluble 2B4 was immobilized on a BIAcore CM5 chip (BIAcore AB, NJ) using either standard amine⁶ or cysteine⁷ coupling chemistry. Kinetic analysis was performed with multiple injections of MHC–peptide over the TCR surface at a flow rate of 15 μl min⁻¹, at 25 °C, on a BIAcore system. Binding data were fitted using the Marquardt–Levenberg nonlinear least-squares algorithm provided in the BIAevaluation 2.1 package. Competitive binding experiments involving empty or 99A-bound E^k were

performed as described⁷. Briefly, detergent-solubilized E^k-MCC was immobilized on a CM5 chip and the TCR was run in solution in the presence of high concentrations of either E^k variant.

Quasi-elastic light scattering. Protein solutions were made in PBS, pH 7.3, and were filtered (Whatman, 0.1 µm) or centrifuged to remove aggregates and dust when necessary. Each solution sample was then injected into a thin-walled cylindrical borosilicate glass cuvette (1-cm diameter) and placed in a vat filled with toluene as the optical matching fluid. During the course of the measurements, the vat temperature was kept at 21 °C. The light source was an argon ion laser (Spectra Physics, $\kappa = 514.4$ nm), and photons scattered by the sample were collected by a photomultiplier tube mounted on the goniometer arm at 90° to the direction of the incident radiation. The photoelectron count-time correlation function was measured with a BI 2030AT (Brookhaven Instruments) digital autocorrelator and analysed using the constrained regularization algorithm, CONTIN²¹. This provides, in the case of a not overly dilute solution of globular particles, an intensity-weighted distribution of translational diffusion coefficients (D) (extracted by means of an inverse Laplace transform). The resulting unimodal or bimodal distributions were characterized by the peak positions. Particle Stokes radii, R_h , were then calculated from the corresponding diffusion coefficients using the Stokes–Einstein relation: $D = kT/6\pi\eta R_h$, in which k is the Boltzmann constant, T is the temperature (in K), η is the solvent viscosity (1.002 cP), and R_h is the hydrodynamic radius of the molecule. The measured time-integrated scattered intensity was calibrated with respect to benzene and, for the binary mixtures analysed, mixing ratios were verified on the basis of intensity measurements of the isolated species.

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A G-protein-coupled receptor for leukotriene B₄ that mediates chemotaxis

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Leukotriene B₄ (LTB₄)¹ is a potent chemoattractant that is primarily involved in inflammation, immune responses and host defence against infection². LTB₄ activates inflammatory cells by binding to its cell-surface receptor (BLTR)³. LTB₄ can also bind and activate the intranuclear transcription factor PPAR α , resulting in the activation of genes that terminate inflammatory processes⁴. Here we report the cloning of the complementary DNA encoding a cell-surface LTB₄ receptor that is highly expressed in human leukocytes. Using a subtraction strategy, we isolated two cDNA clones (HL-1 and HL-5) from retinoic acid-differentiated HL-60 cells. These two clones contain identical open reading frames encoding a protein of 352 amino acids and predicted to contain seven membrane-spanning domains, but different 5'-untranslated regions. Membrane fractions of Cos-7 cells transfected with an expression construct containing the open reading frame of HL-5 showed specific LTB₄ binding, with a K_d (0.154 nM) comparable to that observed in retinoic acid-differentiated HL-60 cells. In CHO cells stably expressing this receptor, LTB₄ induced increases in intracellular calcium, D-myo-inositol-1,4,5-triphosphate (InsP₃) accumulation, and inhibition of adenylyl cyclase. Furthermore, CHO cells expressing exogenous BLTR showed marked chemotactic responses towards low concentrations of LTB₄ in a pertussis-toxin-sensitive manner. Our findings, together with previous reports^{4,5}, show that LTB₄ is a unique lipid mediator that interacts with both cell-surface and nuclear receptors.

Exposure to retinoic acid causes HL-60 human leukaemia cells to differentiate into granulocyte-like cells with properties common to human leukocytes⁶. LTB₄ binding activity in HL-60 cells is increased by 1 µM retinoic acid^{7,8}, and is maximal after 4 days of differentiation (Fig. 1a). We speculated that the increase in LTB₄ binding was due to increased levels of receptor messenger RNA (BLTR mRNA), and therefore attempted to isolate BLTR cDNA from these cells by cDNA subtraction. Following subtraction, candidate cDNAs (Fig. 1b) corresponding to mRNAs induced by retinoic acid were sequenced. Among 66 cDNAs analysed, one (5m-1; Fig. 1c) was found to contain a 267-base-pair (bp) segment of the 3'-untranslated region (3'-UTR) of a gene encoding an orphan seven-span receptor previously isolated from human genomic DNA⁹ and a human B-lymphoblast cDNA library¹⁰. Using this cDNA as a probe, we isolated two full-length cDNA clones (HL-1 and HL-5, Fig. 1c) from a retinoic acid-differentiated HL-60 cDNA library by plaque hybridization.

These two clones contain an identical putative open reading frame (ORF) encoding 352 amino acids, but have 5'-UTRs that are different in both sequence and length (Fig. 1c). The protein encoded by HL-1 and HL-5 is identical to that of the orphan receptor¹⁰. Northern blotting (Fig. 1d) revealed two mRNAs that hybridize to the ORF region of the HL-5 clone in HL-60 and human monocytic leukaemic U-937 cells, both of which have a functional BLTR^{7,8}. Both mRNAs, which may correspond to HL-1 and HL-5, were induced by 1 µM retinoic acid in HL-60 cells (Fig. 1d). It is possible that these mRNAs are differentially transcribed variants of the same gene. Northern blotting of various human tissues revealed that the highest expression of this receptor mRNA is observed in leukocytes,

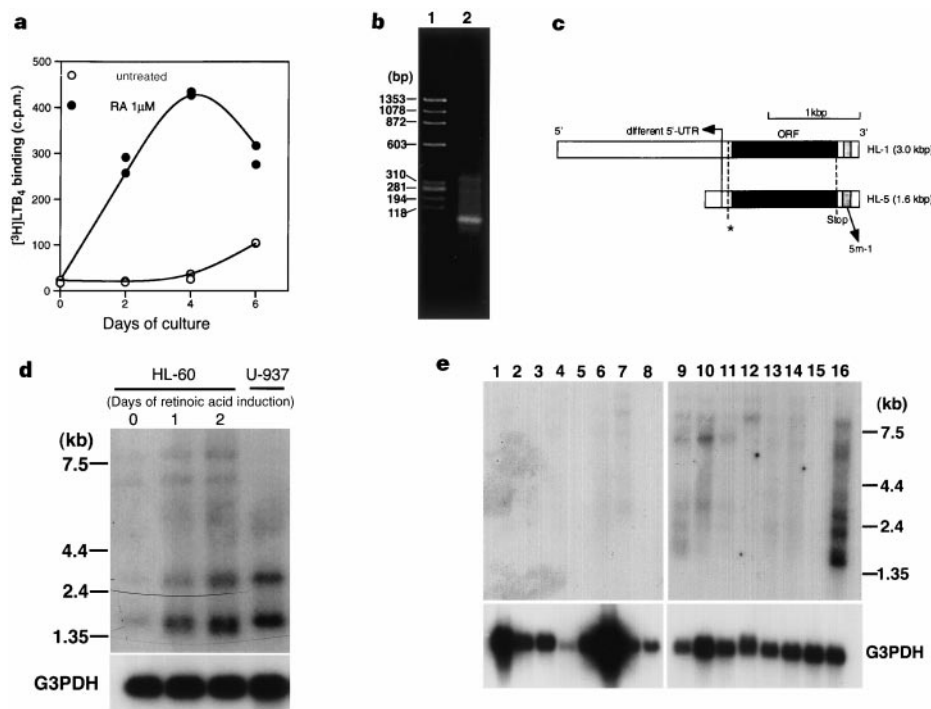


Figure 1 cDNA cloning of a BLTR from retinoic acid-differentiated HL-60 cells. **a**, Specific binding of 0.25 nM [³H]LTB₄ to 5 μg membrane fractions isolated from retinoic acid-treated (filled circles) and nontreated (open circles) HL-60 cells. **b**, Size distribution of cDNAs isolated following subtraction. *Hae*III-digested Φ X 174 DNA (200 ng, lane 1) and an aliquot of subtracted cDNAs (100 ng, lane 2) were electrophoresed in a 2% agarose gel and stained with ethidium bromide. **c**, Primary structures of BLTR cDNAs. The identical open reading frame (ORF, black boxes), different 5'-UTRs (white boxes), in-frame stop codons (*), and the positions of 5m-1 cDNA obtained by subtraction (shaded boxes) are indicated. kbp,

Kilobase pairs **d**, Northern blot analysis of BLTR mRNA in HL-60 and U-937 cells. BLTR mRNAs are induced in the presence of 1 μM retinoic acid in HL-60 cells. Lower panel, hybridization with glutaraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA. **e**, Northern blot analysis of BLTR mRNA expressed in various human tissues. Lanes: 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas; 9, spleen; 10, thymus; 11, prostate; 12, testis; 13, ovary; 14, small intestine; 15, colon; 16, peripheral blood leukocytes. Lower panel, hybridization with G3PDH cDNA.

followed by spleen and thymus, where the expression is much lower (Fig. 1e). The deduced amino-acid sequences of HL-1 and HL-5 showed only low homology with other known proteins. The protein with the highest overall homology was rat somatostatin receptor type 3 (SSTR3)¹¹ (35.8% identity), followed by human SSTR5 (33.1%), human IL-8 receptor (33.0%), human formyl-peptide related receptor II (FMLPR-II, lipoxin A₄ receptor)¹² (30.7%), and human formyl-peptide receptor (FMLPR) (28.6%). Other receptors, including those for prostanoids¹³ and platelet-activating factor¹⁴, showed very weak homology with HL-1 and HL-5, suggesting that the leukotriene receptors may comprise a new receptor family.

An expression vector containing the ORF of HL-5 (pLTBR) was introduced into Cos-7, HEK-293, CHO and C6-15 glioma cells. Cos-7 and HEK-293 cells transiently transfected with pLTBR showed specific binding for 0.25 nM [³H]LTB₄, whereas wild-type cells and cells transfected with the control vector alone did not (Fig. 2a). Membrane fractions of retinoic acid-differentiated HL-60 cells and pLTBR-transfected Cos-7 cells showed similar LTB₄ binding profiles, with *K_d* values of 0.144 ± 0.051 and 0.154 ± 0.054 nM (mean ± s.d., from three separate experiments), respectively (Fig. 2b, c). Two specific BLTR antagonists, ONO 4057¹⁵ and U-75302¹⁶, inhibited [³H]LTB₄ binding, with *K_i* values of about 1.2 μM and 120 nM respectively, in both retinoic acid-differentiated HL-60 cells and pLTBR-transfected Cos-7 cells (Fig. 2d). We next examined the inhibition of [³H]LTB₄ binding by various eicosanoids in the membrane fractions of pLTBR-transfected Cos-7 cells. As shown in Fig. 2e, LTB₄ inhibited [³H]LTB₄ binding most efficiently (*K_i* = 0.38 nM), followed by 20-OH-LTB₄ (7.6 nM), 12-oxo-LTB₄ (ref. 17; 7.6 nM), 12(*R*)-HETE (hydroxyeicosatetraenoic acid) (30 nM), and 20-COOH-LTB₄ (190 nM). These profiles of LTB₄

binding closely match the previous reports for LTB₄ binding in human polymorphonuclear leukocytes¹⁸ and eosinophils¹⁹. These data suggest that the 6-*cis*-configuration, the 12(*R*)-hydroxyl moiety and the methyl group at the C20 position of LTB₄ are all important for binding to BLTR.

We next examined the intracellular signalling stimulated by LTB₄ using CHO cells stably expressing BLTR. We isolated eight CHO clones that responded to 100 nM LTB₄ with calcium mobilization. As all the clones showed similar chemotactic activity towards LTB₄, we chose one of them as a representative (CHO-LTBR) and studied its intracellular signalling in detail. In CHO-LTBR cells, LTB₄ inhibited forskolin-induced adenylyl cyclase activity in a dose-dependent manner, and this inhibition was completely blocked by pretreatment of the cells with 100 ng ml⁻¹ pertussis toxin for 12 h (Fig. 3a). LTB₄ also rapidly increased intracellular calcium concentrations (Fig. 3b) and InsP₃ accumulation (data not shown) in CHO-LTBR cells, and these increases were significantly, but only partially, inhibited by treatment with pertussis toxin (Fig. 3b). These results suggest that BLTR couples to both pertussis-toxin-sensitive and insensitive G proteins in CHO-LTBR cells. LTB₄-dependent increases in intracellular calcium concentration are pertussis-toxin-sensitive in leukocytes²⁰ and differentiated HL-60 cells⁶. In our experiments, LTB₄-evoked calcium increases were completely blocked by pretreatment of retinoic acid-differentiated HL-60 cells with pertussis toxin (data not shown). Different expression patterns of G proteins in CHO and HL-60 cells, or the overexpression of BLTR protein in CHO-LTBR cells, could explain the different sensitivities of the calcium responses to treatment with pertussis toxin. LTB₄-induced hyperadhesiveness of vascular endothelial cells for leukocytes is not affected by pretreatment with 750 ng ml⁻¹ pertussis toxin²¹, suggesting that BLTR couples

to different types of G proteins depending on the type of cell. In fact, higher concentrations of LTB_4 are required for increasing intracellular calcium levels in CHO-LTBR cells (Fig. 3b) compared with retinoic acid-treated HL-60 cells (data not shown).

One of the unique characteristics of LTB_4 is its potent chemoattractant activity²². This phenomenon is important in the initiation and progress of inflammatory reactions. Overproduction of LTB_4 leads to severe inflammatory responses, and high levels of LTB_4 are observed in affected tissues in patients with psoriatic skin²³, inflammatory bowel diseases, and encephalomyelitis²⁴. Migrating leukocytes are known to play a role in each of these disorders. We examined the chemotactic activities of CHO-LTBR cells, and found that they showed prominent chemotaxis toward nanomolar concentrations of LTB_4 (Fig. 4a). By contrast, cells transfected with the control vector did not show such a response (Fig. 4b). The dose dependency of LTB_4 had a bell-shaped pattern, with an optimum concentration of 1–10 nM (Fig. 4c). When LTB_4 was added to both upper and lower chambers at the same concentration, the cells showed significant chemokinetic activities, indicating that LTB_4 can induce both chemotaxis and chemokinesis in CHO-LTBR cells. Exogenous expression of the platelet-derived growth factor (PDGF)-receptor- β induces chemotaxis towards PDGF in CHO cells²⁵. Tyrosine phosphorylation of PLC γ and PI(3)K kinase is required for PDGF-induced chemotaxis. On the other hand, the

mechanisms of chemotaxis mediated by G-protein-coupled receptors remain obscure. LTB_4 -induced chemotaxis was completely inhibited by pretreatment of the cells with 100 ng ml⁻¹ pertussis toxin for 12 h (Fig. 4c). This inhibition is not due to nonspecific toxic effects, because LTB_4 induced robust increases in intracellular calcium in these cells following pretreatment with pertussis toxin (Fig. 3b). Thus, pertussin-toxin-sensitive molecules are apparently necessary for LTB_4 -induced chemotaxis. We also examined the effects of two distinct BLTR antagonists on chemotaxis, and found that U-75302 is more effective than ONO 4057 (Fig. 4d), as in the binding experiments (Fig. 2d). Taken together, our results indicate that clone HL-5 encodes a biologically functional BLTR. Furthermore, they indicate that CHO cells contain the components required for chemotaxis and should therefore be a good tool for analysing the molecular mechanisms of chemotaxis.

A putative purinoceptor, P2Y7, has been described that responds to micromolar concentrations of ATP ($K_i = 37$ nM) and its analogues when expressed in Cos-7 cells²⁶. The primary structure of P2Y7 is identical to that of HL-5. In our experiments, Cos-7 cells were found to contain intrinsic purinoceptors and showed increases in intracellular calcium concentrations following exposure to 1 μM ATP (data not shown). To determine whether BLTR also functions as a purinoceptor, we established stable transformants of pLTBR in C6-15 glioma cells, which possess negligible amounts of intrinsic

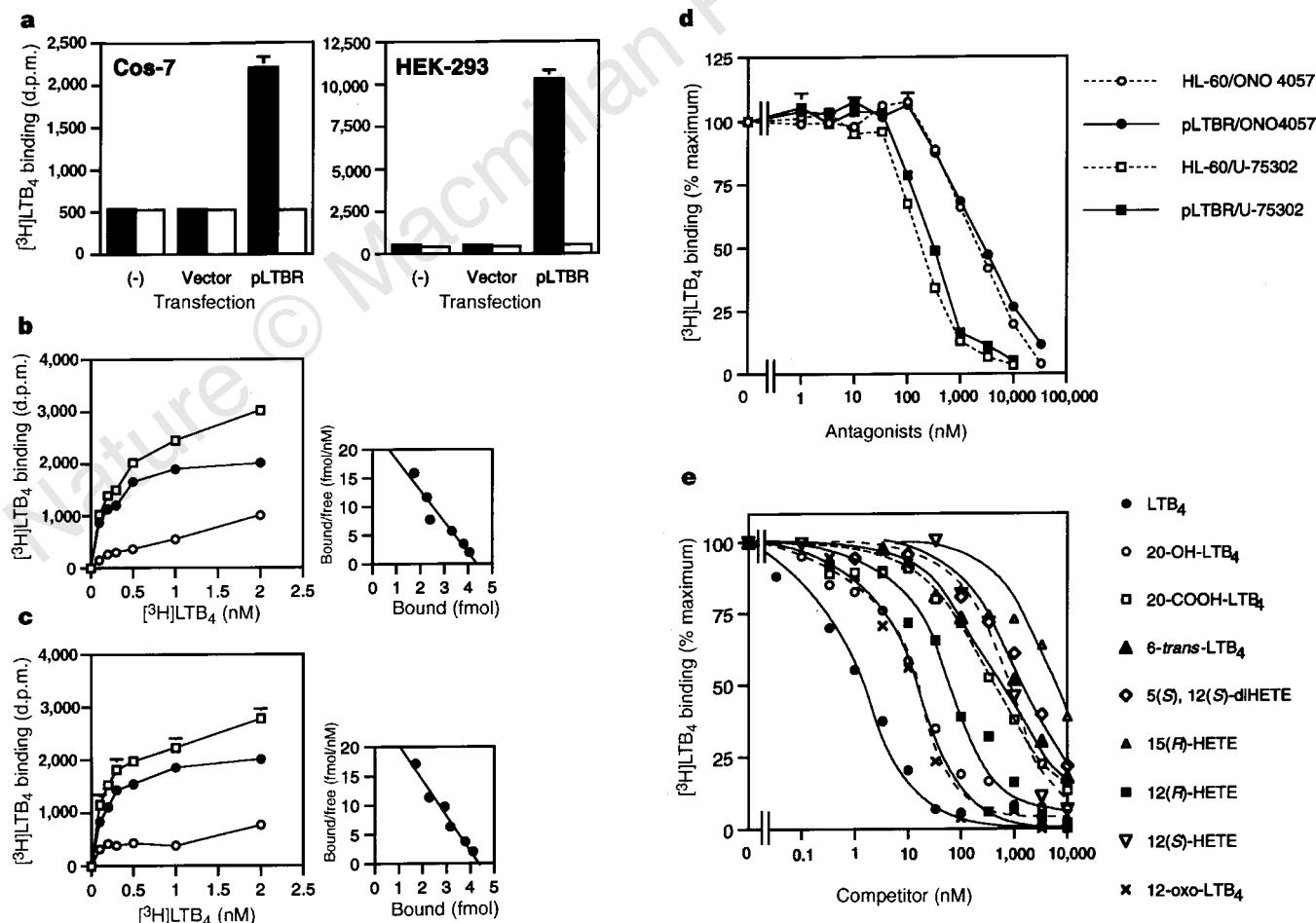


Figure 2 $[^3\text{H}]\text{LTB}_4$ binding to the membrane fractions of retinoic acid-treated HL-60 cells, and Cos-7 and HEK-293 cells transiently transfected with pLTBR. **a**, 0.25 nM $[^3\text{H}]\text{LTB}_4$ binding to the membrane fractions (10 μg) of parental cells (-), control vector (vector) or BLTR expression vector (pLTBR)-transfected Cos-7 and HEK-293 cells. Total binding (black columns) and nonspecific binding (white columns) are presented (mean $\pm$ s.e., $n = 4$). **b, c**, Binding isotherms and Scatchard analyses of $[^3\text{H}]\text{LTB}_4$ binding to the membrane fractions of retinoic acid-

treated HL-60 cells (5 μg , **b**) and pLTBR-transfected Cos-7 cells (10 μg , **c**) ($n = 4$). **d, e**, Inhibition of 0.25 nM $[^3\text{H}]\text{LTB}_4$ binding to the membrane fraction of pLTBR-transfected Cos-7 cells by two LTB_4 antagonists (**d**) and various eicosanoids (**e**) (mean $\pm$ s.e., $n = 4$). The IC_{50} values of the following eicosanoids were over 10 μM : PGD₂, PGE₂, PGF_{2 α} , 5(S)-HETE (hydroxyeicosatetraenoic acid), 5(R)-HETE, 15(S)-HETE, LTD₄, and 5-oxo ETE³⁰.

purinoceptors²⁷. In these C6-15-LTBR cells, ATP up to 300 μ M caused no change in intracellular calcium levels, but significant increases in the calcium concentrations were induced by exposure to 10 nM LTB₄ (data not shown). C6-15 cells transfected with the control vector did not respond to either ATP or LTB₄. These results show that this receptor is not a purinoceptor, but a BLTR.

In conclusion, we have isolated cDNAs encoding a functional BLTR using a cDNA subtraction strategy. CHO cells expressing the receptor showed a potent chemotactic response and activation of intracellular signalling following exposure to LTB₄. These findings should aid in clarifying the onset and progress of various inflammatory diseases in which leukocytes are involved, and in designing BLTR antagonists as anti-inflammatory drugs. Furthermore, our results, together with previous findings^{4,5}, show that a single signalling molecule can bind to both cell-surface and intracellular

receptors. Tissue distribution and K_d values⁴ of these receptors are different. Thus, it is possible that the cell-surface receptor BLTR in leukocytes is responsible for initiating an inflammatory response and that the PPAR α nuclear receptor is for its termination. It will be interesting to determine whether this dual receptor system is limited to LTB₄, or whether it also exists for other lipid mediators and/or more hydrophilic ligands. □

Methods

cDNA subtraction and library screening. HL-60 cells were cultured in RPMI 1640 medium containing 10% FCS. To induce granulocyte-like differentiation, cells were seeded at a density of 10^6 cells ml⁻¹, and cultivated for the indicated number of days in medium containing 1 μ M retinoic acid⁸. Two cDNA pools were synthesized from poly(A)⁺ RNAs isolated from undifferentiated and retinoic acid-differentiated HL-60 cells. The two cDNA pools were digested

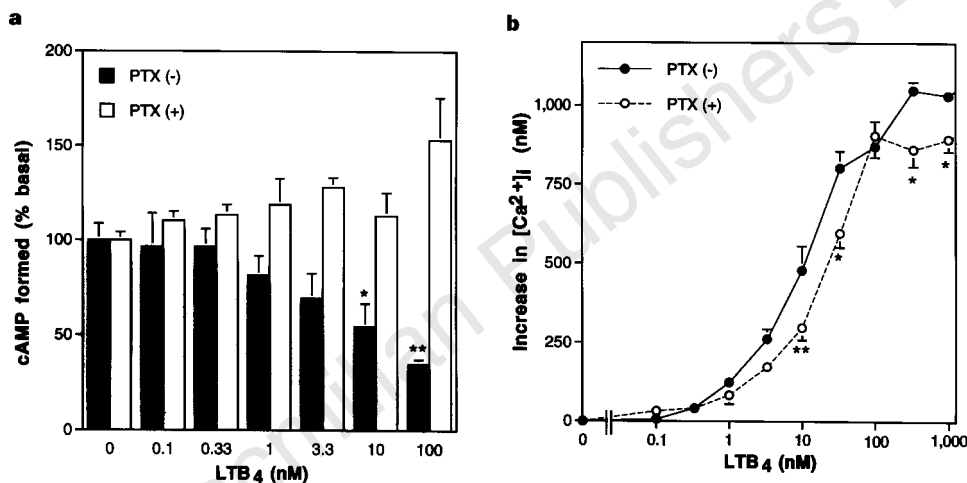


Figure 3 Signal transduction in CHO-LTBR cells. **a**, Cyclic AMP accumulation in forskolin-activated CHO-LTBR cells (mean $\pm$ s.e., $n = 3$). LTB₄ inhibited 0.5 nM forskolin-induced adenylyl cyclase activities (black columns). This inhibition was abolished by pretreatment of the cells with 100 ng ml⁻¹ pertussis toxin (PTX) for 12 h (white columns). The forskolin-induced cAMP levels in the absence of LTB₄ were 983 ± 148 pmol per 10^6 in untreated cells and 472 ± 36 pmol per 10^6 in PTX-

treated cells. Statistically significant differences between control cells treated with forskolin alone, and cells treated both with forskolin and LTB₄ are indicated (* $P < 0.05$, ** $P < 0.01$, unpaired t -test). **b**, Increase in intracellular calcium concentrations in CHO-LTBR by LTB₄ (mean $\pm$ s.e., $n = 3$). Statistically significant differences between control and PTX-treated cells are indicated (* $P < 0.05$, ** $P < 0.01$, unpaired t -test).

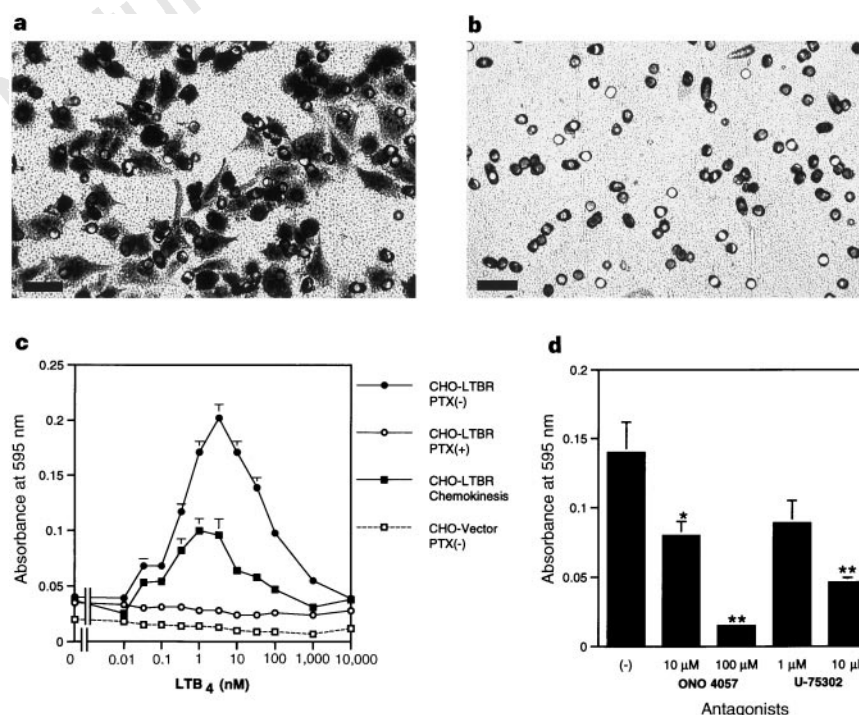


Figure 4 LTB₄-induced chemotaxis in CHO-LTBR cells.

a, b, Light microscope analysis of the migration of the cells towards LTB₄ in the Boyden chamber assay (scale bars, 50 μ m). 1 nM LTB₄ induced potent chemotaxis in CHO-LTBR cells (**a**), but not in control-vector-transfected cells (**b**). Only the pores (8 μ m) in the filter are seen in **b**. **c**, Dose dependency of LTB₄ in chemotaxis and the effect of PTX treatment (mean $\pm$ s.e., $n = 4$). LTB₄-induced chemotaxis (black circles) and chemokinesis (black squares) in CHO-LTBR cells are presented. CHO-LTBR cells pretreated with 100 ng ml⁻¹ PTX for 12 h (white circles), and control vector-transfected cells (white squares) showed no chemotactic activities. **d**, Effects of LTB₄ antagonists on chemotaxis toward LTB₄ (10 nM) in CHO-LTBR cells (mean $\pm$ s.e., $n = 4$). Statistically significant differences are indicated (* $P < 0.05$, ** $P < 0.01$, unpaired t -test).

with *RsaI* before cDNA subtraction. cDNA prepared from RNA isolated on day 3 of induction was subtracted using cDNA prepared from undifferentiated cell RNA using a polymerase chain reaction (PCR)-select cDNA subtraction kit (Clontech). The remaining cDNAs were randomly subcloned into a T-vector (Promega). Sixty-six clones were sequenced, and their sequences were compared with those in the GenBank/EMBL/DBJ database. One clone (5m-1) was found to encode the 3'-UTR of a previously isolated seven-span orphan receptor⁹. The cDNA corresponding to the ORF of the orphan receptor was amplified by PCR from 1 µg of human genomic DNA. The primers used for PCR were 5'-CGGGATCCCCGATGGCGTCAGGAAACCCTTG-3' (sense), and 5'-GGAATTCCTAGTTCAGTTCGTTTAACTTG-3' (antisense). The PCR conditions were as follows: denaturation at 96 °C for 1 min, annealing at 55 °C for 1 min, elongation at 72 °C for 3 min; 30 cycles. The amplified fragment was randomly labelled with [³²P]dCTP, and was used to screen an HL-60 cDNA library, which was constructed in λ Zap-II (Stratagene) from 5 µg poly(A)⁺ RNA of HL-60 cells differentiated by exposure to 1 µM retinoic acid for 3 days. 5 × 10⁵ independent clones were screened and five clones (HL-1 to HL-5) were isolated by high-stringency washing. DNA sequencing revealed that HL-1 and HL-5 contain identical full-length ORFs. The ORF of HL-5 was subcloned in the mammalian expression vector pcDNA3 (Invitrogen), and the resulting plasmid designated pLTBR.

Northern blot analysis. Poly(A)⁺ RNA (3 µg) from HL-60 and U-937 cells was electrophoresed in a 1% agarose gel, and transferred to a Hybond-N nylon membrane (Amersham). Human multiple tissue northern blot filters I and II were purchased from Clontech. The filters were hybridized with [³²P]dCTP-labelled ORF of the HL-5 clone or a human glutaraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA (Clontech) in hybridization buffer containing 4 × SSC, 5 × Denhardt's solution, 0.2% SDS, 200 µg ml⁻¹ salmon sperm DNA, 50% formamide at 42 °C for 24 h. The filters were washed in 0.1 × SSC, 0.1% SDS at 65 °C and subjected to autoradiography.

Expression in mammalian cells and characterization. Cos-7, HEK-293 and C6-15 cells were cultured in DMEM, and CHO cells in F12 medium. Both media contained 10% fetal calf serum. DNA transfection was performed by lipofection using Transfectam (Gibco BRL) for Cos-7, HEK-293, and CHO cells²⁸, or a calcium phosphate method for C6-15 glioma cells²⁷. The membrane fractions were prepared as described¹⁴. Stable transformants were selected with 1 g l⁻¹ Geneticin (Gibco BRL), and cloned by limiting dilution. Clones that showed increases in intracellular calcium following exposure to 100 nM LTB₄ were selected for further analysis. LTB₄ binding assay and measurements of cAMP, InsP₃, and intracellular calcium were carried out using established protocols^{19,28}.

Chemotaxis assay. Polycarbonate filters with 8-µm pores (Neuroprobe) were coated with 13.3 µg ml⁻¹ fibronectin (Sigma) in PBS for 60 min²⁹. A dry coated filter was placed on a 96-blind well chamber (Neuroprobe) containing the indicated amounts of LTB₄, and the CHO cells (200 µl, 8 × 10⁴ per well) were added to the top wells. The ligand solution and cell suspension were prepared in the same buffer (F-12 medium containing 0.1% BSA). After incubation at 37 °C in 5% CO₂ for 4 h, the filter was disassembled. The cells on the filter were fixed with methanol and stained with a Diff-Quick staining kit (International Reagents Corp.). The upper side of the filter was then scraped free of cells. The number of cells that migrated to the lower side was determined by measuring optical densities at 595 nm using a 96-well microplate reader Model 3550 (Biorad).

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The three-dimensional structure of aquaporin-1

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The entry and exit of water from cells is a fundamental process of life. Recognition of the high water permeability of red blood cells led to the proposal that specialized water pores exist in the plasma membrane¹. Expression in *Xenopus* oocytes and functional studies

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of an erythrocyte integral membrane protein of relative molecular mass 28,000, identified it as the mercury-sensitive water channel, aquaporin-1 (AQP1)². Many related proteins, all belonging to the major intrinsic protein (MIP) family, are found throughout nature³. AQP1 is a homotetramer containing four independent aqueous channels⁴⁻⁶. When reconstituted into lipid bilayers, the protein forms two-dimensional lattices with a unit cell containing two tetramers in opposite orientation⁷⁻¹⁰. Here we present the three-dimensional structure of AQP1 determined at 6 Å resolution by cryo-electron microscopy. Each AQP1 monomer has six tilted, bilayer-spanning α -helices which form a right-handed bundle surrounding a central density. These results, together with functional studies, provide a model that identifies the aqueous pore in the AQP1 molecule and indicates the organization of the tetrameric complex in the membrane.

MIP-related proteins all have six stretches of hydrophobic sequence that are suggestive of six transmembrane helices. In addition, these proteins share an NPA (Asn-Pro-Ala) motif in each of the two most prominent loops³. The membrane topology has been confirmed by epitope insertion studies of AQP1 which showed the amino- and carboxy-termini to be on the cytoplasmic side of the membrane¹¹. Site-directed mutagenesis of the loops containing the NPA motifs further indicated that these segments probably line the path of water permeation. These results led to the hourglass model, in which the NPA loops project from the outer and inner leaflets towards the centre of the membrane⁶.

Figure 1a shows the unit cell calculated from 11,054 diffraction intensities and 13,734 phases measured over a tilt angle range of 60° (Table 1). The AQP1 tetramer in the centre is viewed from the extracellular side, whereas adjacent tetramers, which have the opposite orientation, are seen from the cytosolic side. The sidedness has been determined by surface relief reconstruction from metal-shadowed samples and atomic force microscopy of native membranes¹². Figure 1b shows a vertical slice containing four AQP1 monomers overlaid by the relief reconstruction. The cytoplasmic side of the tetramer protrudes further from the membrane than the extracellular side, and the surface relief between tetramers defines the boundaries of the bilayer (marked by a two-headed arrow in Fig. 1b). Narrow vertical clefts between monomers span the membrane (arrows in Fig. 1b). These gaps define the molecular boundaries within the tetramer. A loop (marked by an asterisk in Fig. 1a) links adjacent monomers on the cytoplasmic side, but helix-helix contacts in the narrow cleft may also contribute to the unusual stability of AQP1 tetramers. The square-shaped tetramers are rotated slightly about their fourfold axes, probably to provide the contacts between adjacent tetramers required for crystallization.

Different views of an AQP1 monomer (Fig. 2a-d) reveal six distinct tilted rods of variable lengths that form a right-handed bundle. Their shapes and dimensions suggest that these rods are membrane-spanning α -helices, in agreement with the sequence-based structure prediction (Fig. 2e). The six helices surround a complex central density X, as illustrated by the molecule viewed from the extracellular side in Fig. 2a.

The helix assignment in Fig. 2 is based on several features in the three-dimensional map. First, the C terminus has been identified as the most prominent cytosolic protrusion by comparing surface reliefs of metal-shadowed crystals before and after digestion with carboxypeptidase Y¹². As this protrusion is close to the fourfold axis, helix 6 should be in this region (Fig. 2a). Rotating the monomer by 45° clockwise around the x-axis (Fig. 2b) unveils the end of helix 6 which is seen to protrude away from the AQP1 molecule, thus supporting its assignment as the C terminus. The loop of the next helix to the right in Fig. 2b extends to the adjacent monomer (asterisk in Fig. 1a) and is likely to be the N terminus of AQP1. At the other end, helix 1 is connected to helix 2 by way of the density indicated by an arrow in Fig. 2b, consistent with the short loop A (~12 residues). This connection becomes clearer when the molecule is

contoured to include a larger volume. Helix 2 continues on the cytoplasmic side into a loop projecting back into the centre of the monomer, most probably loop B. The predicted loop D is very short (~6 residues), and is represented by a bulge on the cytoplasmic side (Fig. 2c, arrow). No contiguous density could be assigned to the long loop C between helices 3 and 4. This region coincides, however, with a prominent protrusion of about 5 Å on the extracellular side observed by AFM¹². In addition, the surface topography measured with the AFM supports the right-handed α -helical bundle presented here.

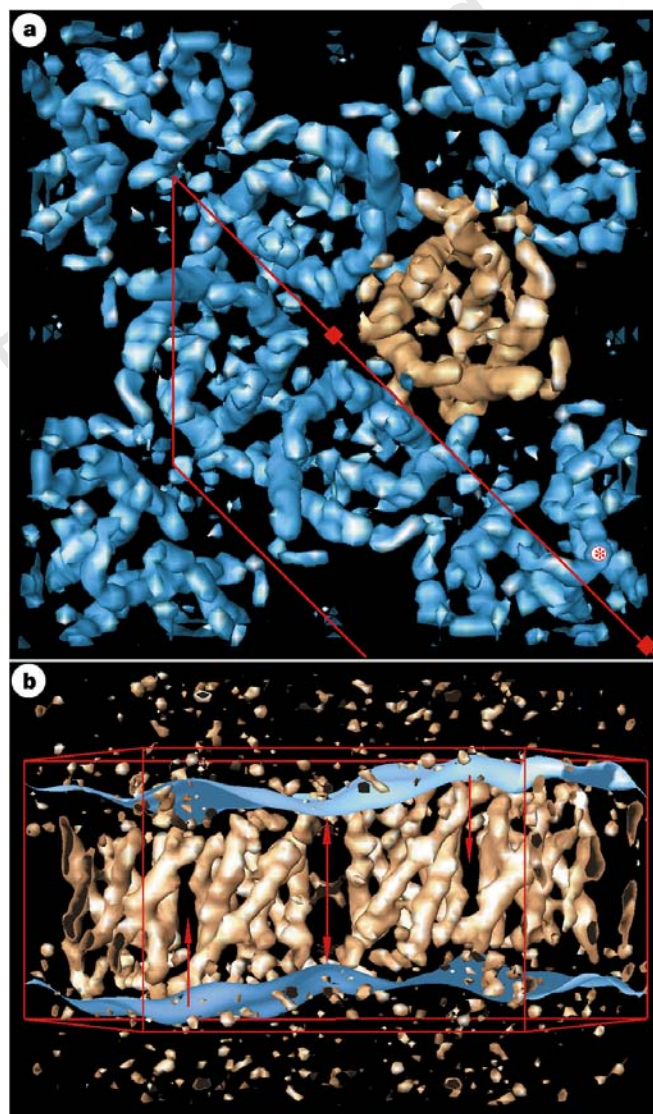


Figure 1 The unit cell of two-dimensional AQP1 crystals has a side length of 96 Å and houses eight asymmetric units that form two tetramers integrated into the bilayer in opposite orientations. **a**, The view along the fourfold symmetry axis (♦) shows the extracellular surface of the central tetramer, with one monomer coloured in gold. Tetramers adjacent to the central tetramer are seen from the cytoplasmic side, which exposes the connecting loop between monomers (red asterisk). Molecular boundaries are reflected by narrow gaps marked by red arrows in the vertical slice displayed in **b**. This slice, with a width of 152 Å and a thickness of 30 Å, contains four monomers and has been cut as outlined in red in **a**. The overlaid surface reconstruction determined by metal-shadowing and atomic microscopy¹² illustrates that AQP1 protrudes significantly from the membrane on the cytosolic side. In addition, the surface extends down to the lipid bilayer between tetramers as indicated by a two-headed arrow.

Table 1 Crystallographic data

Symmetry	P4 ₂ 2
Lattice constants	$a = b = 96 \text{ \AA}$, $c = 100 \text{ \AA}$ (assumed) $\alpha = \beta = \gamma = 90^\circ$
Electron diffraction	
Number of diffraction patterns	47 (0°, 5; 20°, 10; 45°, 11; 60°, 21)
Resolution limit for merging	6.0 Å
Number of merged intensities	11,054
R_{merge}	0.403*
R_{Friedel}	0.406*
Completeness	97.8% (0°–60°) 86.2% (0°–90°)
Images	
Number of images	63 (0°, 7; 20°, 10; 45°, 15; 60°, 31)
Resolution limit for merging	6.0 Å
Number of merged phases	13,734
Phase residual†	31.0° (100–6 Å) 46.6° (8–6 Å) 28.4° (15–8 Å) 20.1° (100–15 Å)
Completeness	99.3% (0–60°) 87.5% (0–90°)

* High because of poor electron statistics.

† Phase residuals were calculated from 6,854 reflections with an $\text{IQ} \leq 7$ (ref. 20).

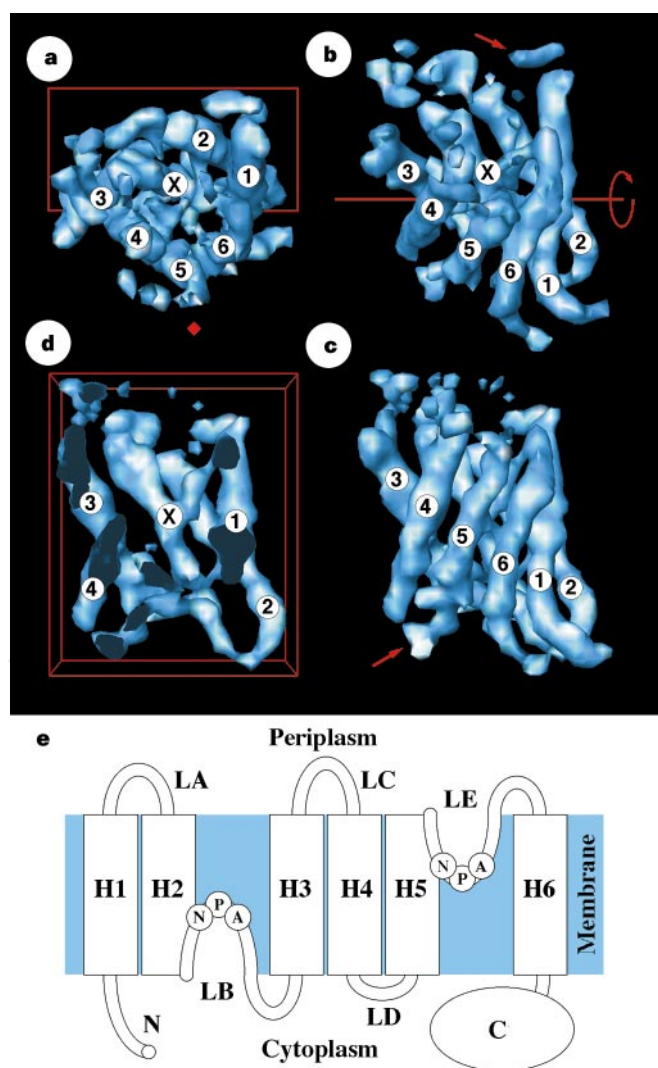


Figure 2 Top, AQP1 monomer as seen **a**, from the extracellular side, and **b**, after a clockwise rotation around the x-axis by 45°. Bottom (from right to left), the monomer after another 45° clockwise rotation around the x-axis (**c**), and cut open to expose the central density X (**d**). The slice shown in **d** is outlined in **a**, and it is 16 Å thick. The red diamond marks the fourfold axis in **a**, and the horizontal line in **b** indicates the x-axis. The sequence-derived prediction of the AQP1 structure is displayed in **e**, and specifies the helix and loop labels.

In Fig. 2d, helices 5 and 6 were cut away to show density X. This unusual structure is wide on the extracellular side, has a side arm and seems to end above the prominent loop connected to helix 2. Although loops connected to density X are not clear for helices 5 and 6 on the extracellular side, the widening of the density towards this end suggests connections on that side. Therefore we propose that density X contains loop E with one of the NPA motifs. Loop B with the other NPA motif is seen to extend from helix 2 on the cytoplasmic side of the molecule. The hourglass model positions these densities in close proximity to the water channel.

An extensive spectroscopic comparison of AQP1 with bacteriorhodopsin has suggested that AQP1 is an all-helical protein with an α -helix content that is consistent with six membrane-spanning segments of 17–22 residues¹³. Furthermore, the measured dichroic ratio has indicated that the helices are tilted with respect to the normal of the bilayer by an average of 21°.

We conclude that the present helix assignment is a reasonable working hypothesis. The right-handed twist in the helix bundle is also a feature commonly observed in soluble proteins¹⁴. We propose that the central mass is formed by the extended loops B and E which carry the highly conserved residues involved in water permeation. Much-higher-resolution information is required to gain insight into the tantalizing specificity of AQP1 for water. □

Methods

Two-dimensional crystals of AQP1 were produced as described¹⁵ and prepared for cryo-electron microscopy by addition of trehalose to a final concentration of 3–15%. The crystal suspension was applied to a molybdenum electron microscope grid coated with a flat thin carbon film. The grid was blotted with filter paper, immediately plunged into liquid ethane and transferred into a JEOL JEM-3000SFF electron microscope. The JEM-3000SFF, which is equipped with a liquid helium-cooled stage¹⁶ and a field emission electron source, was operated at an acceleration voltage of 300 kV. Electron micrographs were recorded under low dose conditions (<20 electrons $\text{\AA}^{-2}$) at nominal magnifications of $\times 50,000$ on Kodak SO163 film and developed with Kodak D19 developer. The images and electron diffraction patterns were taken at a stage temperature of 4.2K. Micrographs displaying well-ordered two-dimensional arrays were selected by optical diffraction and areas of $6,000 \times 8,000$ pixels were digitized with a Scitex LeafScan 45 (ref. 17) using a step size of 5 μm . Images were processed on DEC/Alpha workstations with a modified version of the MRC image processing suite¹⁸. After two cycles unbending and correction for the contrast transfer function, 63 images were averaged to generate a merged phase data set. Electron diffraction data were recorded with a GATAN 2K $\times$ 2K slow-scan CCD camera connected to an Apple Macintosh computer. Forty-seven electron diffraction patterns were evaluated to calculate the amplitudes, which were combined with the image phases to produce the final three-dimensional electron-density map. Density maps were isocontoured on an SGI workstation using a marching cube algorithm¹⁹.

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Three-dimensional organization of a human water channel

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Aquaporins (AQP) are members of the major intrinsic protein (MIP) superfamily of integral membrane proteins and facilitate water transport in various eukaryotes and prokaryotes^{1,2}. The archetypal aquaporin AQP1 is a partly glycosylated water-selective channel^{3,4} that is widely expressed in the plasma membranes of several water-permeable epithelial and endothelial cells^{2,5}. Here we report the three-dimensional structure of deglycosylated, human erythrocyte AQP1, determined at 7 Å resolution in the membrane plane by electron crystallography of frozen-hydrated two-dimensional crystals^{6,7}. The structure has an in-plane, intramolecular 2-fold axis of symmetry located in the hydrophobic core of the bilayer. The AQP1 monomer is composed of six membrane-spanning, tilted α -helices. These helices form a barrel that encloses a vestibular region leading to the water-selective channel, which is outlined by densities attributed to the functionally important NPA boxes⁸ and their bridges to the surrounding helices. The intramolecular symmetry within the AQP1 molecule represents a new motif for the topology and design of membrane protein channels, and is a simple and elegant solution to the problem of bidirectional transport across the bilayer.

Projection density maps of AQP1 (originally called CHIP28, for channel-forming integral protein, relative molecular mass 28K)⁹ have been determined by electron cryocrystallography^{7,10,11}. These analyses differed in the medium used for specimen cryopreservation, the level of glycosylation, the source of the protein, and the nominal resolution of the maps. The overall features observed in all three projection maps were similar, but the interpretation in terms of the possible secondary structure varied. To delineate further the secondary and tertiary structure in detail, we have determined the

three-dimensional density map of deglycosylated, human erythrocyte AQP1 at 7 Å resolution by electron cryocrystallography (Fig. 1a). The unperturbed structure in the bilayer was visualized by analysing minimal-dose images and diffraction patterns recorded from frozen-hydrated two-dimensional crystals⁶ tilted by up to 45° in the electron microscope. To our knowledge, this is the highest-resolution structure of a membrane protein determined by electron crystallography for a specimen preserved in vitrified buffer.

AQP1 monomers pack as tetramers ($\sim 60 \times 60 \text{ Å}^2$ in the bilayer plane) in a tetragonal crystalline lattice⁷ (two-sided plane group symmetry, $p4_212$; $a = b = 99.6 \pm 0.5 \text{ Å}$). Each monomer is approximately cylindrical with a diameter of $\sim 30 \text{ Å}$ and a height of $\sim 60 \text{ Å}$. The three-dimensional density map shows that the prominent feature in each monomer is a group of six identifiable, tilted ($18\text{--}30^\circ$), approximately cylindrical rods (A–F) which form a barrel surrounding a vestibular region (Fig. 1). The dimensions of these rods ($36\text{--}44 \text{ Å}$ long and $\sim 7 \text{ Å}$ in diameter) are similar to those for transmembrane α -helical segments seen in three-dimensional density maps of bacteriorhodopsin¹² and LHC II (ref. 13) calculated at comparable resolution. We therefore interpret these rods of density as α -helices that form a six-helix barrel (Fig. 1). Such a six-span transmembrane arrangement is consistent with the general topology proposed for the MIP family¹⁴, as well as that for AQP1 (ref. 15), which has the N and C termini on the cytoplasmic side. Although α -helices tend to pack with a left-handed twist, the six-helix barrel displays a right-handed twist (Fig. 1). As a consequence of the significant tilt of the helices, the densities observed in the previous projection maps^{7,10,11} do not correspond to single helices, but rather to regions where adjacent helices overlap. The six tilted α -helices within each monomer appear to pack in three two-helix pairs (A–B, C–D and E–F; Fig. 2). Within a pair the observed packing angle for the two helices is $20\text{--}35^\circ$ (Fig. 1b, c), which is common for α -helices¹⁶. Neighbouring monomers in a tetramer interact through helices that are packed as tightly as helices within individual monomers (Figs 1a and 2a). Further, densities for adjacent monomers overlap on one side of the bilayer (Fig. 2b), suggesting interactions between monomers that may stabilize the tetramer. This density implies an asymmetric disposition of the monomer with respect to the bilayer, and is interpreted as mass residing on the cytoplasmic face¹⁷. We therefore attribute this density to the C and N termini, as well as the interhelical loops from adjacent monomers.

Non-crystallographic pseudo-2-fold symmetry is observed in the AQP1 monomer (Figs 1b, 2a and 3), which is consistent with the well-known tandemly repeating motif in AQP1 and the MIP family^{18,19}. This 2-fold rotation axis is located $\sim 3 \text{ Å}$ away from the putative centre of the bilayer (Fig. 1b), is inclined by 8° to the a (or b) unit cell axis, and passes through the crystallographic 4-fold rotation axis (Fig. 2a). This symmetry (Fig. 3) was suggested by visual inspection of the three-dimensional density map, and was confirmed by calculation of the rotation function (see Methods). Because the N and C termini are both located on the cytoplasmic side of the bilayer²⁰, this non-crystallographic symmetry would apply only to the transmembrane domains. Counting the six major hydrophobic segments sequentially from the N terminus^{14,15}, the homologous segments 1 and 4, 2 and 5, and 3 and 6 are oriented in opposite directions. This is a consequence of the six-span arrangement of the AQP1 polypeptide chain¹⁵ and is consistent with the observed in-plane pseudo-2-fold symmetry. The pseudo-2-fold symmetry also imposes strong constraints on models for the transmembrane topology of AQP1. Taking into account the two possible vectorial orientations of the AQP1 molecule in the synthetic lipid bilayer, the number of arrangements of the six transmembrane segments in the three-dimensional density map is reduced drastically from 1,440 ($2 \times 6!$) to 96 ($2 \times 2^3 \times 3!$).

Within the six-helix barrel, the pseudo-2-fold axis passes through a central block of density, which appears to be connected to the

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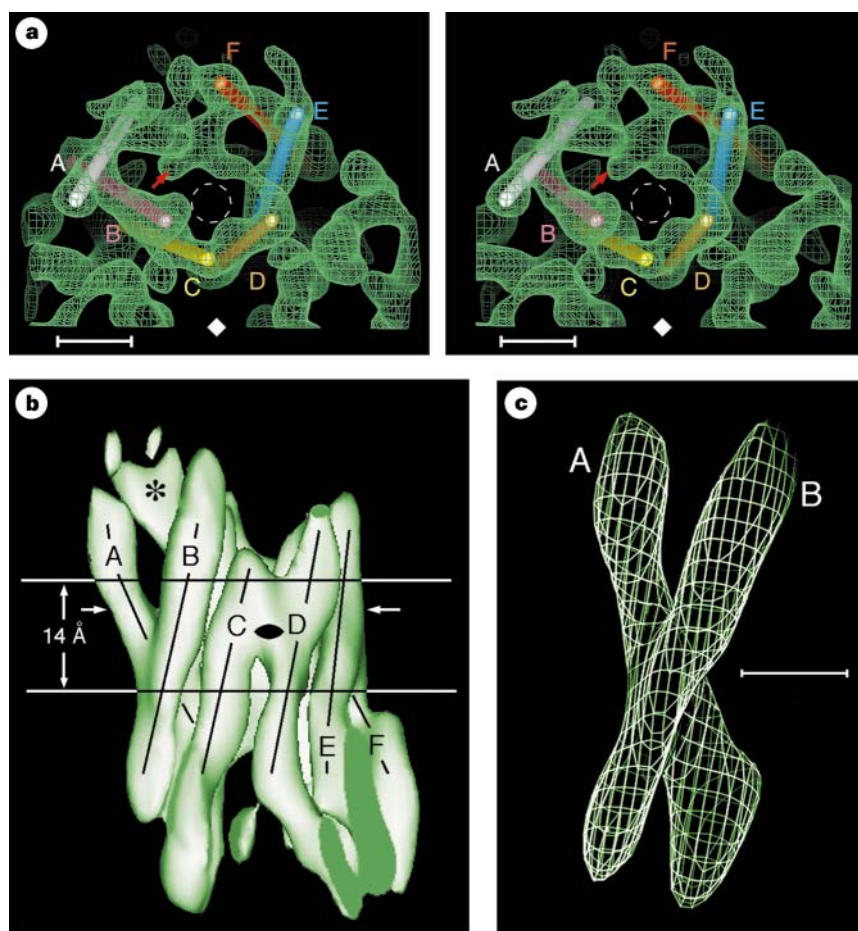


Figure 1 a, Stereo pair of the three-dimensional density map of frozen-hydrated, deglycosylated, human erythrocyte AQP1, viewed approximately perpendicular to the bilayer, showing one monomer and portions of adjacent monomers within a tetramer. The rods trace the approximate paths of the centres of six tilted helices (A to F) that form a barrel surrounding a vestibular region, indicated by the dashed circle. The arrow indicates the density assigned to the vertically apposed NPA boxes. Also visible are portions of densities suggesting linkages of the NPA boxes to the surrounding protein. **b**, A surface-shaded representation of the six-helix barrel viewed parallel to the bilayer with the lines indicating the approximate helix axes. Molecular pseudo-2-fold symmetry (indicated by the black ellipse) is strongest (Fig. 3) within the demarcated transmembrane region. The asterisk indicates a portion of density bridging the NPA box to helix F. The arrows identify the plane containing the phase origin (putative centre of the bilayer) in the *z*-direction. **c**, An example of the arrangement of α -helices in one two-helix pair (packing angle 25°) viewed roughly perpendicular to the bilayer. Figures were rendered at twice the standard deviation of the map density using AVS software²⁸ for **a** and **b**, and the program O²⁹ for **c**. Scale bar, 10 Å.

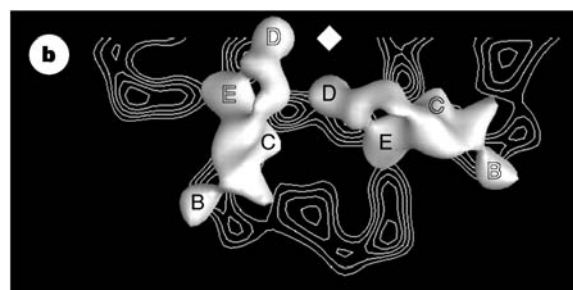
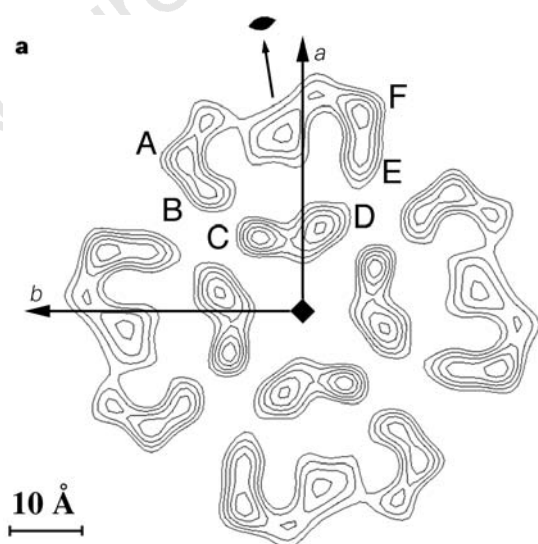


Figure 2 Intra- and inter-monomer interactions. **a**, Packing of helices seen in a contoured section through the three-dimensional density map viewed perpendicular to the bilayer. Intra-monomer interaction is indicated by individual tightly packed two-helix pairs A-B, C-D and E-F. Inter-monomer interaction within the bilayer is suggested by the similarity in the distances of separation between neighbouring helices from adjacent monomers and those between adjacent helices belonging to different helix pairs in a monomer. This section contains the in-plane, non-crystallographic, pseudo-2-fold rotation axis, indicated by the arrow. **b**, Surface-shaded representation of the extra-membrane density. We interpret that this density located at the extremities of helices B, C, D and E of adjacent monomers resides on the cytoplasmic face¹⁷, and is composed of the charged C and N termini and hydrophilic interhelical loops. The viewing direction, corresponding to the distal side in Fig. 1a, is opposite to that in **a**.

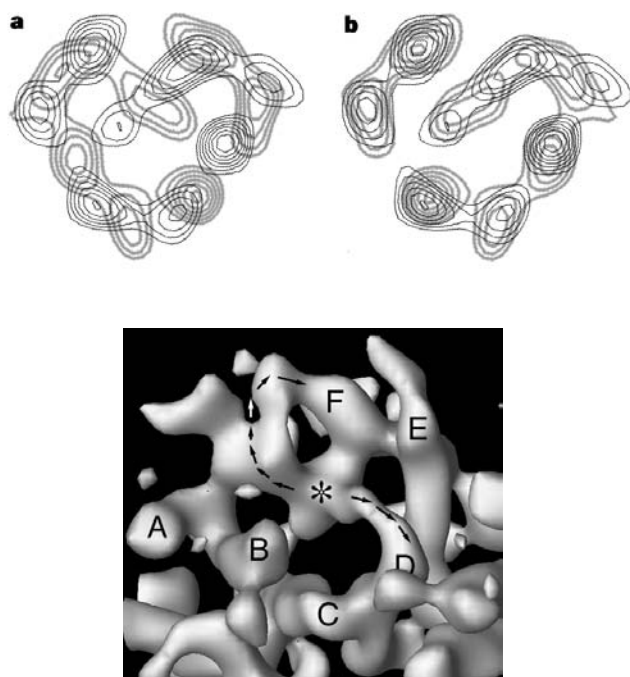


Figure 3 Demonstration of the in-plane pseudo-2-fold symmetry in the AQP1 monomer. The light and grey contours represent density of a monomer in two planes sectioned parallel to the bilayer that are each 7 Å away from the plane containing the pseudo-2-fold axis indicated in Fig. 1b. **a**, There is little overlap of the density contours when these two sections are superimposed. **b**, There is strong overlap in the locations and the strengths of the density features after the application of the 2-fold rotation to one of the sectioned planes.

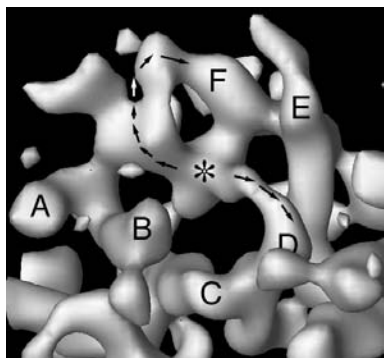


Figure 4 A surface-shaded representation showing an example of bridging density that suggests linkages of the central block (asterisk) to helices D and F. The letters indicate extremities of the helices. Viewing direction is approximately perpendicular to the bilayer and similar to that in Fig. 1a. This figure was rendered at twice the standard deviation of the map density using AVS software²⁸.

surrounding protein barrel (Fig. 1a). In the published projection maps^{7,10,11}, the density for this block varied significantly relative to that for the surrounding protein. We found that the strength of this density in projection was sensitive to the amplitudes of very low-resolution (>24 Å) reflections obtained from electron diffraction patterns. In our three-dimensional map, synthesized with the larger volume of tilt data, this density was observed consistently. Two sets of bridges of density related by the pseudo-2-fold symmetry suggest linkages of this central block to the surrounding barrel. One set, located on the proximal side as viewed in Fig. 1a, is directed towards helices F and D (Fig. 4); the other, located on the distal side as viewed in Fig. 1a, is directed towards helices A and C. The bridging densities to helices A and F are strong and cylindrically shaped (~12 Å long), whereas those to helices C and D are weaker and irregularly shaped (Figs 1a and 4). These bridges and the block of density reside in the interior of the protein. They are therefore likely to represent additional, predominantly hydrophobic segments of the polypeptide chain, such as those containing the conserved asparagine-proline-alanine (NPA) boxes⁸ (that is, the loops connecting helices 2 and 3 and 5 and 6, on the opposite sides of the bilayer). We note that several features of the three-dimensional map greatly restrict the number of possible models for AQP topology. These are the constraints imposed by the pseudo-2-fold symmetry, the putative linkages identifying helices 2 and 3, or 5 and 6, and the asymmetric disposition of AQP1 (ref. 17) (Fig. 2b) in the bilayer.

As observed in the earlier projection maps^{7,10,11}, the AQP1 monomer encloses a vestibule (Fig. 1a) which has a diameter of ~8 Å at the narrowest point. The pathway for selective water transport may be enclosed by this vestibule, which is outlined by the putative NPA boxes, portions of interhelical loops in the interior of the protein, and by the helices near the 4-fold axis. Studies to investigate which regions of the AQP1 sequence are important for function have focused primarily on the interhelical loops⁸ containing the NPA boxes. Amino-acid side chains of the α -helices lining this vestibule are not visible at the current resolution but may also be involved in conferring selective permeability.

The six-helix barrel of AQP1 is likely to be a characteristic folding motif in the aquaporin family because of the high sequence homology. The three-dimensional structure of AQP1 may therefore provide a framework for experiments to determine key residues in the channel region that confer specificity for solute transport in the

aquaporins. More important, our results reveal a novel intramolecular 2-fold symmetry in a membrane protein (Fig. 3). In this case, an intragenic duplication event has resulted in a channel that transports water in either direction. Such coupling between tandem repeats in sequence and 2-fold symmetry in the membrane plane provides a simple and elegant solution to the problem of bidirectional transport across the bilayer. This contrasts with the asymmetric functional properties of ligand- and voltage-gated channels²¹. Our results, therefore, establish a new motif for the topology and design of membrane protein channels. □

Methods

Data collection. Two-dimensional crystallization of purified, deglycosylated, human erythrocyte AQP1 has been described previously^{6,22}. Preparation of frozen-hydrated specimens and recording of minimal-dose images and electron diffraction patterns from crystals tilted up to 45° were performed as reported previously⁷, except that molybdenum instead of copper electron-microscope grids were used. We processed 29 images and 18 electron diffraction patterns; all but 10 of the processed images (nominal magnification of $\times 50,000$ and underfocus of -860 to 12,700 Å) were recorded using a Philips CM200FEG electron microscope operated at 200 kV; the remainder of the images and all diffraction patterns were collected using a Philips CM12 electron microscope operated at 100 kV.

Data processing and analysis. The programs used for processing of the images and diffraction patterns were developed by R. Henderson *et al.*²³. High-resolution images were corrected for lattice distortions, effects of the contrast transfer function and astigmatism. Only peaks up to an in-plane resolution of 7 Å were considered in the computed Fourier transform because of the non-isotropic fall-off of signal in the image of a tilted crystal, especially at high tilt angles. Furthermore, only those peaks with amplitudes more than 1.6 times the background r.m.s. levels ($IQ \leq 6$, in the nomenclature of Henderson *et al.*²³) were included in the analysis. For the electron diffraction patterns, a reflection was excluded if the average of the intensities of the Friedel pairs was less than their difference and greater than 5 times the standard deviation of such averages for all reflections in that pattern. A total of 3,752 sampled phases calculated from the images (averaged merging error per image = 23.6°) and 4,828 sampled amplitudes generated from the diffraction patterns were included in the analysis. Least-squares fitting of the lattice lines²⁴ was performed using the phases derived from the images and the amplitudes derived from the diffraction patterns. This fit yielded an overall weighted phase residual of 21.2°. The smoothly fitted curves were sampled at intervals of 0.005 Å⁻¹ to generate

structure factors, which were then used to calculate the three-dimensional density map. Corresponding to the in-plane resolution of 7 Å, a vertical resolution of ~20 Å was estimated for the three-dimensional density map from the point spread function²⁵. The absolute hand of the map was determined by ensuring that the angle of tilted views of the two-dimensional crystal was recorded with the correct sign. As a check for the assignment of the sign of the tilt angles, we used two-dimensional crystals of bacteriorhodopsin (purple membrane) for which the hand is known. Images recorded from tilted, glucose-embedded purple membrane samples were subjected to the same protocol as our analysis of AQP1, and the resulting phases were compared with the reported phases for purple membrane²⁶. Search for non-crystallographic symmetry was performed by calculating the rotation function using the program POLARREN in the CCP4 package²⁷. A non-crystallographic peak in the membrane plane was consistently seen for all resolution ranges (data not shown). This peak corresponds to a 2-fold rotation axis inclined by 8° with respect to the unit cell *a* (or *b*) axis and has the maximum strength for a radius of integration of 40 Å.

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Crystal structure of the complex between human CD8αα and HLA-A2

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The dimeric cell-surface glycoprotein CD8 is crucial to the positive selection of cytotoxic T cells in the thymus¹. The homodimer CD8αα or the heterodimer αβ stabilizes the interaction of the T-cell antigen receptor (TCR) with major histocompatibility complex (MHC) class I/peptide by binding to the class I molecule². Here we report the crystal structure at 2.7 Å resolution of a complex between CD8αα and the human MHC molecule HLA-A2, which is associated with peptide. CD8αα binds one HLA-A2/peptide molecule, interfacing with the α2 and α3 domains of HLA-A2 and also contacting β₂-microglobulin. A flexible loop of the α3 domain (residues 223–229) is clamped between the complementarity-determining region (CDR)-like loops of the two CD8 subunits in the classic manner of an antibody–antigen interaction, precluding the binding of a second MHC molecule. The position of the α3 domain is different from that in uncomplexed HLA-A2 (refs 3, 4), being most similar to that in the TCR/Tax/HLA-A2 complex⁵, but no conformational change extends to the MHC/peptide surface presented for TCR recognition. Although these shifts in α3 may provide a synergistic modulation of affinity, the binding of CD8 to MHC is clearly consistent with an avidity-based contribution from CD8 to TCR–peptide–MHC interactions.

Soluble forms of recombinant human CD8αα homodimer (residues 1–120) and HLA-A2/pol (residues 1–276 of the heavy chain, 1–99 of β₂-microglobulin (β₂m) and the Pol peptide epitope³ comprising HIV-1 reverse transcriptase residues 309–317) associated under crystallization conditions at pH 6.5 to yield crystals that contained one CD8αα/HLA-A2/peptide complex per crystallographic asymmetric unit. The complex structure was determined by molecular replacement and refined to a crystallographic R-value of 20.6% for all data between 15.0 Å and 2.65 Å resolution. Crystallographic statistics are reported in Table 1. All parts of the complex are well ordered, with the exception of residues 27–30 in one subunit and 115–120 in both of the CD8α subunits (see Methods).

The overall structure of the CD8αα/HLA-A2/peptide complex is shown in Fig. 1a. The box-like CD8αα homodimer provides two approximately orthogonal surfaces to bind separately regions on the α2 and α3 domains of HLA-A2 (Fig. 1b–d). The only substantial difference from the structures of the uncomplexed HLA-A2/pol peptide³ and CD8αα homodimer⁶ is a shift in the position of the HLA-A2 α3 domain (Fig. 2a). As noted previously from comparison of isolated MHC class I molecules, the α3 domain can vary in its position relative to the rest of the molecule⁷. Superpositions of isolated molecules indicate a fan-like continuum of possible positions covering 3.5°; the α3 domain position in the complex

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structure represents an extreme lying some 7.0° from the mean of these orientations (Fig. 2a). The contribution of the CD8 α subunits to the binding is asymmetric. Both subunits (designated CD8 α -1 and CD8 α -2) make interactions through all of their CDR-like loops with the $\alpha 3$ domain (Fig. 1c and Table 2), and subunit CD8 α -1 makes additional use of β -strand A in contacting the HLA-A2 $\alpha 2$ domain and β_2m (Fig. 2d and Table 2). These interactions bury 131, 711 and $206 \text{ \AA}^2$ of solvent-accessible area from HLA-A2 $\alpha 2$, $\alpha 3$ and β_2m , respectively, matched by 686 and $261 \text{ \AA}^2$ from CD8 α -1 and CD8 α -2. For the dominant HLA-A2 $\alpha 3$ /CD8 α interface, a single loop region (residues 223–229) provides the major contribution from HLA-A2 $\alpha 3$, pinned between the CD8 α subunits by both main-chain and side-chain interactions. HLA-A2 $\alpha 2$ and CD8 α -1 form a looser association with few direct protein–protein interactions. The interactions are primarily electrostatic (polar atoms contribute 80% of the area buried on the HLA-A2 $\alpha 2$) and are limited to side chains. The interaction of CD8 α -1 with residues 58–

60 in the DE loop of β_2m is also limited to side-chain contacts. There are no potential glycosylation sites within any of the interfaces. With the exception of slight alterations in conformation and a reduction in flexibility of the CDR-like loops, the structure of CD8 α is unchanged from that of the uncomplexed dimer⁶. Particular care was taken during crystallographic refinement of the complex to establish unambiguously the nature of the disulphide bonds in the CD8 α subunits. The canonical disulphide bond of the immunoglobulin fold (C22 to C94 for CD8 α) is formed, at full occupancy, in both subunits. This is in agreement with the disulphide arrangement observed in the uncomplexed human CD8 α crystal structure⁶. By analogy with biochemical data for murine CD8 (ref. 8), an alternative disulphide arrangement (C22 to C33) may be adopted in the α -subunit of CD8 $\alpha\beta$. The canonical bond is clearly favoured energetically in homodimerized CD8 α , being independent of the mode of protein production or of interaction with MHC class I.

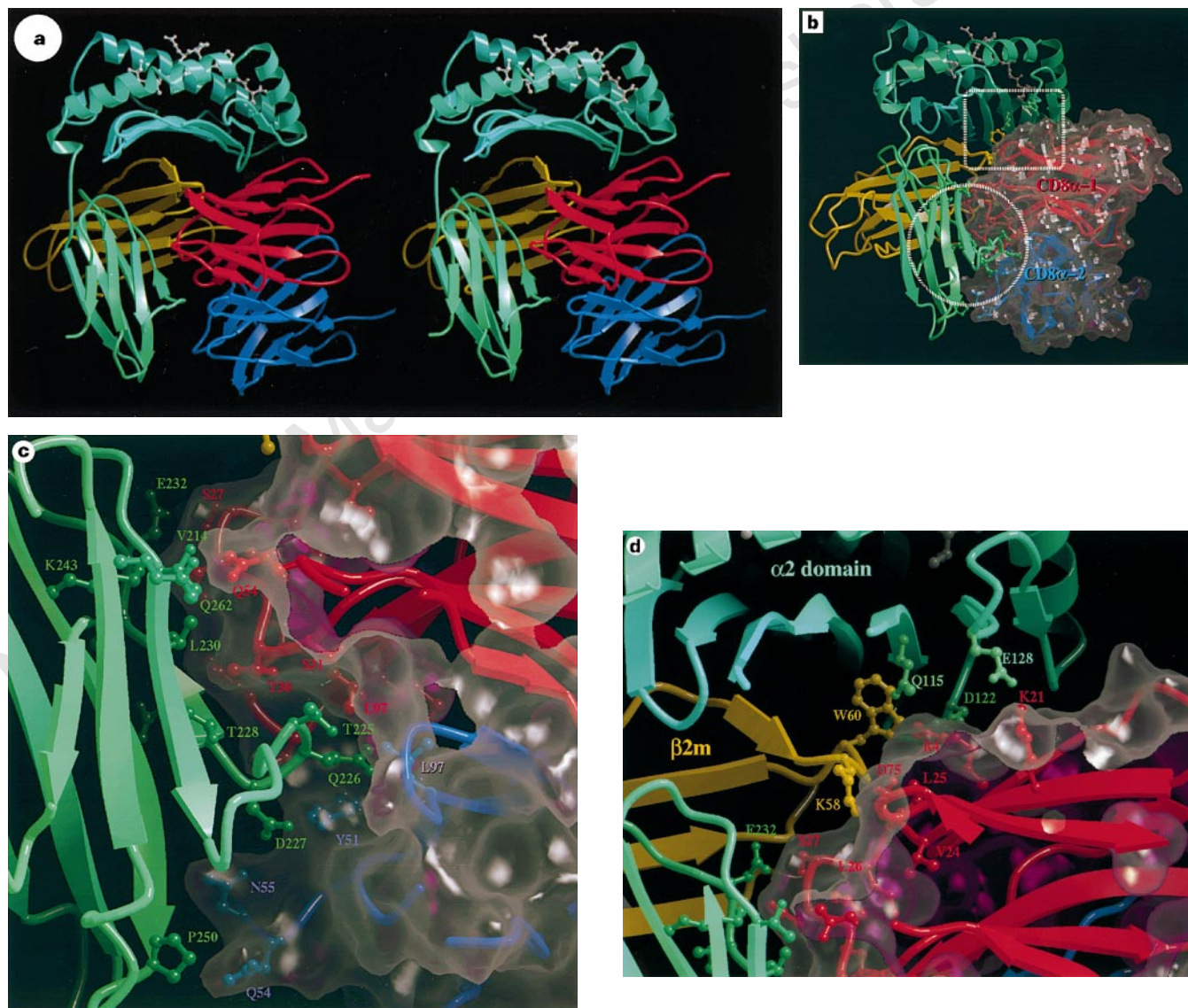


Figure 1 The structure of the CD8 α /HLA-A2/peptide complex and the interaction surfaces. **a**, Stereo view of the complex. The HLA-A2 heavy chain (green), β_2m (gold), CD8 α -1 (red) and CD8 α -2 (blue) are depicted schematically, and the polypeptide (ILKEPVHGV) is shown in white as a ball-and-stick representation. Secondary structure assignments are from program DSSP²⁴, modified in places to accord with previous reports. This orientation defines a standard view for **b–d**. **b**, The interaction surfaces. The solvent-accessible surface of CD8 α (calculated in the absence of the rest of the complex by the program VOLUMES;

R. Esnouf, unpublished) is shown as a semi-transparent surface. The regions depicted in close up in **c** and **d** are indicated by a circle and a rectangle, respectively. The polypeptide chains are represented schematically as in **a** but the view is slightly rotated about the horizontal. **c**, The HLA-A2 $\alpha 3$ /CD8 α interaction surface. **d**, The HLA-A2 $\alpha 2$, β_2m and CD8 α -1 interaction surface. Components of this figure and of Fig. 2 were drawn using programs MOLSCRIPT²⁵ (with modifications by R. Esnouf) and RASTER3D²⁶;

Table 1 Data collection and refinement statistics

Crystal space group:	$P2_12_12_1$
Cell parameters (Å):	$a = 70.9, b = 89.6, c = 116.0$
Data processing	
Observations to 2.65 Å	77,731
Unique reflections	21,379
Completeness (%)	96.5 (96.4)
I/σ	15.2 (2.6)
$R_{\text{merge}} (\%)^*$	9.5 (44.6)
Refinement	
Data range (Å)	15.0–2.65
Reflections ($F > 0$)	21,207
Completeness (%)	96.7 (96.2)
Reflections in R_{free} set	1,639
Non-hydrogen atoms	5067
Solvent molecules	108
R.m.s. Δ bond lengths (Å) [†]	0.006
R.m.s. Δ bond angles (deg) [†]	1.26
R.m.s. Δ B -factors for bonded atoms (Å ²) [†]	2.1
R.m.s. Δ for n.c.s. equivalent atoms (Å) [‡]	0.039
R.m.s. Δ for n.c.s. B -factor restraints (Å ²) [‡]	0.73
$R_{\text{free}} (\%)^{\S}$	27.6
$R_{\text{cryst}} (\%)^{\parallel}$	20.6

Values in parentheses correspond to the highest-resolution shell (2.71–2.65 Å).
^{*} $R_{\text{merge}} = \sum |I - \langle I \rangle| / \sum I$
[†]Root-mean-squared deviations (r.m.s. Δ) in bond length and angles are given from ideal values.
[‡]Root-mean-squared deviations in position and B -factor for the two CD8 subunits related by non-crystallographic symmetry (n.c.s.) refer to those residues (66 of 114) that were restrained during refinement.
[§] $R_{\text{free}} = \sum ||F_o| - |F_c|| / \sum |F_o|$
^{||} R_{free} is as for R_{cryst} but calculated for a test set comprising reflections not used in the refinement.

The various data^{9–11} that have implicated residues of the $\alpha 2$ and $\alpha 3$ domains of MHC class I in CD8 recognition may now be reassessed in the context of the crystal structure (Table 2). In particular, mutagenesis data¹⁰ have highlighted the importance of residues 223–229 of the MHC class I $\alpha 3$ domain. These residues form a prominent loop at the surface of the MHC class I molecule, and contribute the main single component of the interface with CD8 α . In the absence of CD8 binding, this loop is highly flexible⁷, whereas in the CD8 α /HLA-A2/peptide complex, the loop is clamped between the CDR1-like and CDR3-like loops from both of the CD8 subunits (Fig. 1c), imparting rigidity while conserving the basic conformation (Fig. 2b). CD8 α resembles an immunoglobulin F_v-like homodimer², and this mode of binding is analogous to that used by the variable domains of antibodies when binding to antigenic surfaces containing a single prominent loop¹². Residues from the β -strands of the HLA-A2 $\alpha 3$ domain make additional, peripheral interactions with the CDR2-like loops of both CD8 subunits and the CDR1 and CDR3-like loops of CD8 α -1 (Fig. 1c and Table 2). Point mutations at several of these β -strand residues did not have a great effect on CD8 binding¹⁰; this concentration of a few residues critical to the interaction energy in the central portion of a larger contact area is similar to the functional interaction surfaces characterized for other protein–protein recognition events¹³. Although the interface between HLA-A2 $\alpha 2$ and CD8 α -1 is less intimate, it nevertheless seems to be critical for stable interactions. Mutagenesis of any of the three $\alpha 2$ domain residues, Gln 115, Asp 122 and Glu 128, which make direct contacts in the interface (Fig. 1d and Table 2), abolishes CD8 binding¹¹. All the HLA-A2 residues that mediate important interactions with CD8 α are non-polymorphic, which is consistent with the largely allele-independent nature of CD8 binding.

One human allele, HLA-Aw68, has been reported to show an aberrantly low level of CD8 binding⁹, an effect that has been linked to polymorphism at residue 245 (A245V) (ref. 9). Although there is no direct contact between CD8 α and residue 245, mutation of this residue in HLA-Aw68 (ref. 14) leads to a significant distortion of the 223–229 loop from the conformation seen in all other structurally characterized alleles and required for binding to CD8 (Fig. 2b). The apparent origin of this change is a reorientation of the loop to avoid

Table 2 Interactions between CD8 α and HLA-A2 in the complex

Hydrogen bonds		
CD8 α	HLA-A2*	Length (Å)
CD8 α -1		
R4 N η 1	Q115 O ϵ 1	3.2
R4 N η 2	D122 O δ 1	3.0
R4 N ϵ	D122 O δ 2	3.0
K21 N ζ	E128 O ϵ 1	2.9
K21 N ζ	E128 O ϵ 2	2.8
S27 O γ	E232 O ϵ 1	2.7
N28 O δ 1	K243 N ζ	3.2
T30 O γ 1	T225 O	2.7
Q54 O ϵ 1	Q262 N ϵ 2	2.9
N99 O δ 1	L230 N	3.0
N99 N δ 2	L230 O	3.4
S100 O	Q226 N ϵ 2	3.0
S100 O γ	Q226 O	2.7
V24 O	K58(β)N ζ	3.0
D75 O δ 2	K58(β)N ζ	2.7
CD8 α -2		
S34 O γ	Q226 N ϵ 2	3.0
Y51 O η	D227 O δ 2	3.0
Contacts between HLA-A2 and CD8 α (distances < 4.0 Å)		
Structural element	CD8 α	HLA-A2*
CD8 α -1		
β -strand A	R4	K58(β), W60(β), Q115, D122
β -strand B	K21	E128
	V24	K58(β)
CDR-1 like	L25	K58(β)
	L26	K58(β)
	S27	E232
	N28	L230, V231, E232, K243
	P29	L230
	T30	T225, T228, L230
	S31	T225
β -strand C'	Y51	T225
CDR-2 like	Q54	T214, Q262
D-E loop	D75	K58(β)
β -strand F	L97	T225, Q226
CDR-3 like	N99	T228, E229, L230
	S100	Q226
β -strand G	M102	Q226
CD8 α -2		
β -strand C	S34	Q226
β -strand C'	Y51	Q226, D227
CDR-2 like	S53	D227
	Q54	E198
	N55	E198, V248
β -strand F	L97	T225, Q226

*Residues for the HLA-A2 are heavy chain unless marked (β).

steric conflict between the side chain of Thr 228 and the side chain of residue 245, which is enlarged from alanine in HLA-A2 to valine in HLA-Aw68. Even relatively subtle alterations in residues such as 245, which affect the packing of the $\alpha 3$ domain core and hence trigger conformational changes in the 223–229 loop, will influence binding to CD8.

Mutational analyses¹⁵ of CD8 α have indicated that residues in the CDR-like loops and the A and B β -strands are involved in binding to MHC class I. These data are in agreement with the observed structure of the CD8 α /HLA-A2/peptide complex (Table 2). The binding of CD8 $\alpha\beta$ to MHC class I is tighter than that of CD8 α ^{16,17}. CD8 α and β sequences have only ~20% residue identity but may be aligned satisfactorily by taking residue Phe 3 in CD8 α as the amino-terminal residue of CD8 β , and assuming only one insertion of three residues in the CC' loop of CD8 β . Given the highly asymmetric binding of the CD8 dimer to the MHC molecule (73% of the surface area buried by CD8 α on complex formation is from the CD8 α -1 subunit), the contribution of the CD8 β subunit will depend on whether it adopts the role of the CD8 α -1 or CD8 α -2 subunit. A simple scoring of putative interactions highlights additional electrostatically favourable interactions on substituting the β sequence into the CD8 α -2 subunit. These are predicted between two residues (Asn 28 and Arg 30 in CD8 β) of the CDR1-like loop (which remains relatively flexible in the current CD8 α /MHC complex) and residues Asp 223 and

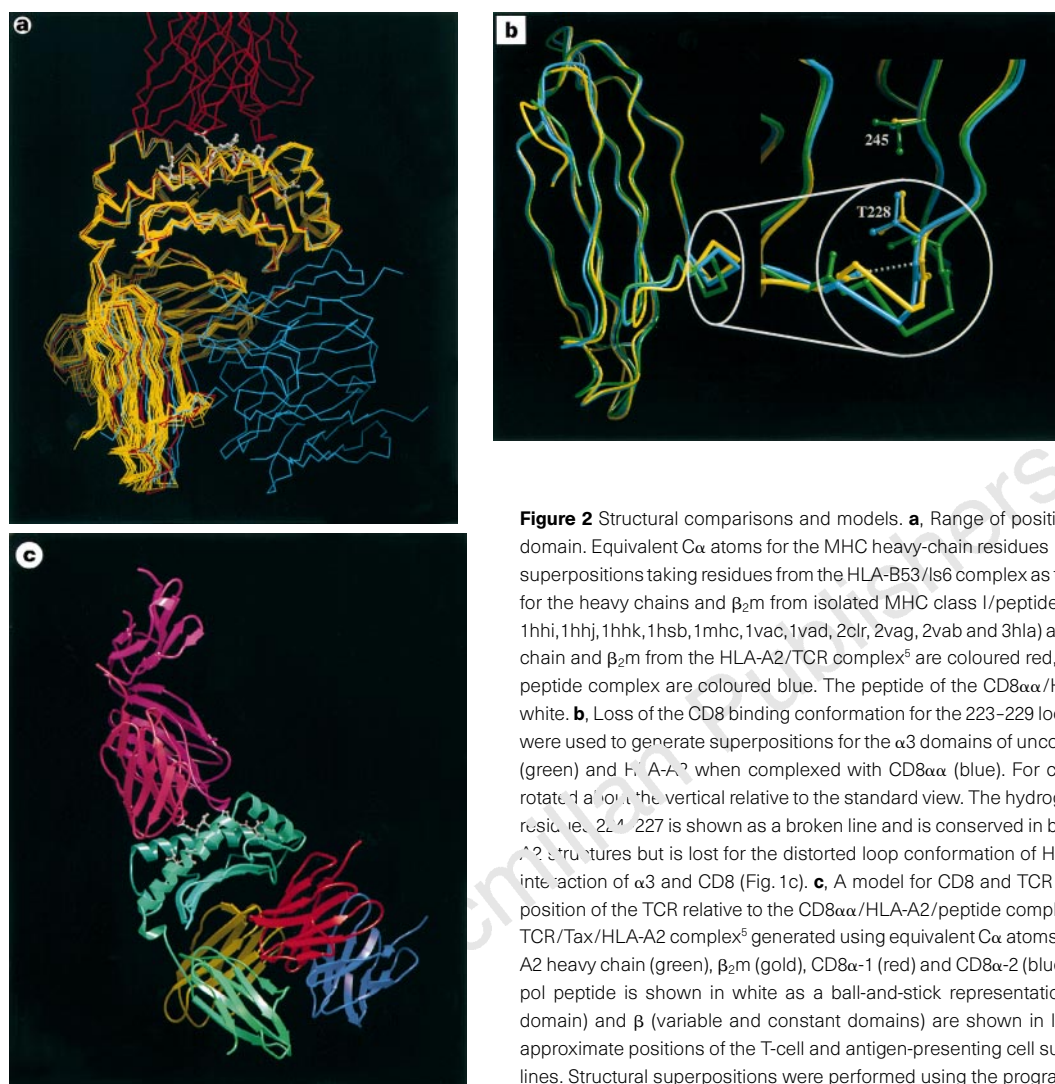


Figure 2 Structural comparisons and models. **a**, Range of positional variation for the MHC class I $\alpha 3$ domain. Equivalent $C\alpha$ atoms for the MHC heavy-chain residues 1–174 were used to generate pairwise superpositions taking residues from the HLA-B53/Is6 complex as the common reference set²⁷. $C\alpha$ traces for the heavy chains and β_2m from isolated MHC class I/peptide complexes (PDB codes: 1hhg, 1hhh, 1hhi, 1hhj, 1hkh, 1hsb, 1mhc, 1vac, 1vad, 2clr, 2vag, 2vab and 3hla) are shown in yellow; the HLA-A2 heavy chain and β_2m from the HLA-A2/TCR complex⁵ are coloured red, and those from the CD8 $\alpha\alpha$ /HLA-A2/peptide complex are coloured blue. The peptide of the CD8 $\alpha\alpha$ /HLA-A2/peptide complex is shown in white. **b**, Loss of the CD8 binding conformation for the 223–229 loop in HLA-Aw68. Equivalent $C\alpha$ atoms were used to generate superpositions for the $\alpha 3$ domains of uncomplexed HLA-A2 (yellow), HLA-Aw68 (green) and HLA-A*0201 when complexed with CD8 $\alpha\alpha$ (blue). For clarity, the right-hand-side close up is rotated 180° about the vertical relative to the standard view. The hydrogen bond defining the β turn between residues 224–227 is shown as a broken line and is conserved in both the isolated and complexed HLA-A2 structures but is lost for the distorted loop conformation of HLA-Aw68. This β turn is central to the interaction of $\alpha 3$ and CD8 (Fig. 1c). **c**, A model for CD8 and TCR binding to MHC class I. The putative position of the TCR relative to the CD8 $\alpha\alpha$ /HLA-A2/peptide complex is based on a superposition of the TCR/Tax/HLA-A2 complex⁵ generated using equivalent $C\alpha$ atoms for HLA-A2 residues 1–174. The HLA-A2 heavy chain (green), β_2m (gold), CD8 α -1 (red) and CD8 α -2 (blue) are depicted schematically, and the pol peptide is shown in white as a ball-and-stick representation. The superposed TCR α (variable domain) and β (variable and constant domains) are shown in light and dark pink, respectively. The approximate positions of the T-cell and antigen-presenting cell surfaces are indicated by broad broken lines. Structural superpositions were performed using the program SHP²⁸.

Asp 227 of $\alpha 3$ as well as between residue Lys56 in the CDR2-like loop and residue Glu 198 at the base of the $\alpha 3$ domain.

The crystal structure of the CD8 $\alpha\alpha$ /HLA-A2/peptide complex demonstrates unambiguously that the stoichiometry of functional binding is 1:1, that is, one MHC class I molecule with one CD8 dimer. Thus, although the structure confirms the prediction, based on surface charge distribution, that the surface of CD8 containing the CDR-like loops would interact with residues 222–229 in the MHC class I molecule⁶, it excludes the possibility that the binding mode facilitates one CD8 dimer forming complexes with two MHC class I molecules¹⁵. Overall, 947 Å² of solvent-accessible area is buried in the complex by CD8 $\alpha\alpha$, matched by 1,048 Å² from the MHC class I molecule. These values are very similar to those reported for the TCR/Tax/HLA-A2 complex⁵ and reflect the comparable reported affinities of the interactions (K_D values of 11–39 μ M for MHC/CD8)¹⁸. Several features of the interaction between MHC and CD8 may underlie this relatively low affinity: the highly polar nature of the interfaces (detailed analyses of several protein–protein interactions have highlighted hydrophobic residues as the major contributors to interaction energy¹³); the entropic cost of reduced mobility in the CDR-like and $\alpha 3$ loops; and the energetic penalty for reorientation of the $\alpha 3$ domain. The $\alpha 3$ domain reorientation is the only significant conformational change of the HLA-A2 in complex with CD8; the closest equivalent orientation for an $\alpha 3$ domain is in the TCR/Tax/HLA-A2 complex⁵ (Fig. 2a). Changes focused at the pivotal point of the $\alpha 3$ movement (Leu 178) could possibly transmit an effect from CD8 binding into the

peptide-binding groove (and vice versa), but no such effect is apparent from the CD8 $\alpha\alpha$ /HLA-A2/peptide complex. If both CD8 and TCR on binding to MHC pay the same, albeit small, energetic cost in moving the $\alpha 3$ domain, then the binding of either would increase the affinity of the other. Irrespective of the significance of this effect, the fact that the binding sites of CD8 and TCR to MHC class I are spatially separate but require similar orientations of the MHC molecule relative to the target cell surface (Fig. 2c) is consistent with an avidity-based contribution from the CD8 co-receptor to MHC–TCR binding. □

Methods

Protein expression, refolding and purification. An extracellular fragment of the human CD8 α chain was expressed in *Escherichia coli* BL21-DE3-pLysS from plasmid BJ112 that encodes residues 1–120 in the T7 expression vector pGMT7. The extracellular portion of HLA-A*0201 (HLA-A2) was similarly expressed in BL21-DE3-pLysS from plasmid BJ075 that encodes residues 1–276 under T7 promoter control in the vector pET23d (Novagen). The β_2m expression plasmid was provided by D. N. Garboczi and D. C. Wiley (Harvard Medical School). The HIV-1 pol peptide epitope, ILKEPVHGV, was synthesised by the Oxford Centre for Molecular Sciences. All proteins were recovered from *E. coli* inclusion bodies by solubilization in 8 M urea buffers by modifications of described protocols^{5,19}. HLA-A2/pol complex and CD8 $\alpha\alpha$ homodimer were refolded individually and purified by gel filtration (Superdex G-75) and cation exchange (Mono S) on a Pharmacia FPLC system.

Crystallization. Crystals were grown by vapour diffusion at room temperature using microbridges from sitting drops containing 1 μ l of protein solution

(20 mg ml⁻¹ HLA A2/pol peptide complex in 10 mM Tris-HCl, 10 mM NaCl, pH 8.0, mixed in 2:1 stoichiometry with 10 mg ml⁻¹ CD8 α in the same buffer) plus 1 μ l of reservoir solution (12% PEG 20000, 100 mM MES pH 6.5). The presence of both HLA-A2 and CD8 α molecules in the crystals was demonstrated by polyacrylamide gel electrophoresis. Crystals were harvested into a modified reservoir solution containing 15% PEG 20000, 50 mM MES, pH 6.5. For cryogenic data collection, crystals were transferred stepwise to harvest buffer supplemented with progressively higher concentrations of glycerol up to a final concentration of 20%, and flash-frozen by plunging into liquid propane. A data set was collected in-house (with crystal maintained at 100 K in a Cryostream; Oxford Cryosystems) using a 30-cm diameter MAR-Research detector mounted on a Rigaku RU200 generator ($\lambda = 1.54 \text{ \AA}$) fitted with Yale mirror optics. Crystals belong to the space group $P2_12_12_1$, with unit cell dimensions $a = 70.9 \text{ \AA}$, $b = 89.6 \text{ \AA}$, $c = 116.0 \text{ \AA}$, and contain one HLA-A2 and one CD8 α dimer per asymmetric unit. The data were auto-indexed and integrated with the program DENZO²⁰ followed by scaling using the program SCALEPACK²⁰ to yield a data set 96.5% complete at $2.65 \text{ \AA}$ ($R_{\text{merge}} = 9.5\%$; Table 1). **Structure determination.** The structure was determined by molecular replacement using the program AMoRe²¹. Search probes consisted of the original HLA-A2 model, which does not contain a peptide antigen (refined at $2.6 \text{ \AA}$; Protein Data Bank accession code 3HLA), and the CD8 α homodimer (refined at $2.6 \text{ \AA}$; Protein Data Bank accession code 1CD8). Unambiguous solutions were found in the cross-rotation and translation functions for both molecules which when combined resulted in an R_{cryst} of 43.4% ($R_{\text{free}} = 45.3\%$) for data between 10.0 to $3.5 \text{ \AA}$. After four-domain rigid-body refinement ($\alpha 1\alpha 2$, $\alpha 3$, $\beta_2\text{m}$ and CD8 α) with X-PLOR, the R_{cryst} dropped to 39.4% ($R_{\text{free}} = 40.2\%$) for data between 15.0 and $3.0 \text{ \AA}$. $2F_o - F_c$ and $F_o - F_c$ electron density maps calculated on the basis of these phases showed clear density for the peptide. These maps also presented poor density for several regions, including some of the CDR-like loops, which were removed from the model and gradually rebuilt during the course of the refinement.

Refinement. Refinement was carried out with standard protocols in program X-PLOR²², maintaining tight non-crystallographic symmetry restraints between the two subunits of CD8 α , and was alternated with manual rebuilding in the interactive graphics program O²³. A bulk solvent correction allowed all measured data from 15.0 to $2.65 \text{ \AA}$ to be used. In the final stages of the refinement, ordered water molecules were added to the model in the interface regions. Refinement statistics are given in Table 1. The refined atomic model of the complex comprises residues 1–114 of both CD8 α subunits, residues 1–276 of the HLA-A2 heavy chain, residues 2–99 of $\beta_2\text{m}$, the 9-residue peptide and 108 ordered water molecules. Of the non-glycine residues, none lie in an energetically disallowed region of the Ramachandran plot. For residues 27–30 in CD8 α -2 the quality of the electron density is poor but sufficient to define unambiguously the course of the polypeptide mainchain for this apparently flexible loop region. No electron density is observed for residues 115–120, which are presumed to form part of an extended stalk in the intact extracellular region of CD8 (ref. 6).

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Correspondence and requests for materials should be addressed to J.L.B. (e-mail: john.bell@ndm.ox.ac.uk) or E.Y.J. (e-mail: yvon@biop.ox.ac.uk). Atomic coordinates for the CD8 α /HLA-A2/peptide complex have been deposited with the Protein Data Bank (Brookhaven National Laboratory), and are available pre-release from yvon@biop.ox.ac.uk and jose@biop.ox.ac.uk.

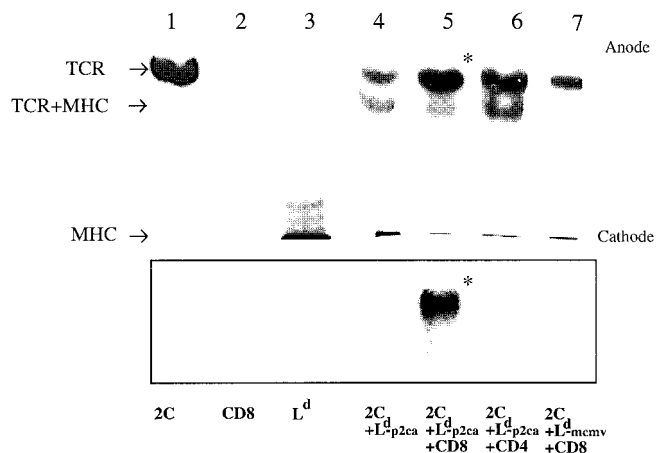
correction

CD8 enhances formation of stable T-cell receptor/MHC class I molecule complexes

K. Christopher Garcia, Christopher A. Scott, Anders Brunmark, Francis R. Carbone, Per A. Peterson, Ian A. Wilson & Luc Teyton

Nature **384**, 577–581 (1996)

No bands were evident in the first three lanes of the gel presented in Fig. 4. The corrected figure is shown here. □



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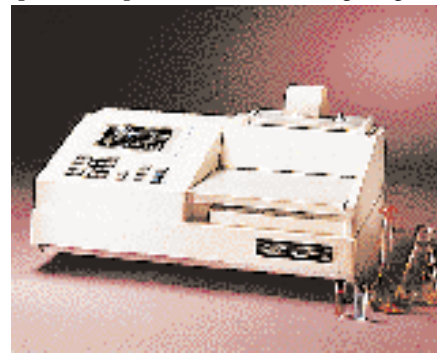
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Series 3000 spectrophotometer

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Scanning ultraviolet/visible instruments designed to combine small size with low cost of purchase and maintenance

All instruments feature a high-resolution, backlit liquid-display screen, which allows spectra or plots to be scrolled, giving an



Cecil Instruments' Series 3000 spectrophotometer.

effective screen width of 430 mm. An encoded software enhancement facility has been included in the design, enabling additional software modules to be activated immediately upon entering a correctly allocated code. Any instrument may be further enhanced, *in situ*, by obtaining this code via telephone, fax or e-mail. The Series 3000 range has a stated optical bandwidth of just 1.8 nm and uses high-quality optics to reduce stray light to a stated 0.01 per cent and achieves a wavelength precision of 0.1 nm. The electronic design facilitates baseline stability of $\pm 0.001 \text{ A h}^{-1}$. Validation software is provided for use in conjunction with sets of filters calibrated to traceable standards and for convenient routine verification of performance. Wavelength and absorbance accuracy, optical bandwidth, stray light or noise levels may be confirmed with results displayed on-screen with a dated and timed report produced automatically. Wavelength scanning at scan speeds that are user selectable from 30–4,000 nm min^{-1} allows spectra to be displayed on-screen with all peaks and valleys clearly annotated for absorbance value and wavelength. A wide range of quantitative methods, including two- and three-wavelength assays, Warburg and Christian assays, wine assays at up to ten wavelengths, Lowry, Bradford, Biuret or BCA assays, may be calculated and stored as secure personal methods.

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This excitation source comprises a xenon light source, galvanometric scanner, blazed grating and mirror optics. The monochromatic light is coupled to specially designed epifluorescent condensers through a solid quartz fibre (the condensers are available for most microscopes). The unit delivers a bright (4–8 mW), evenly illuminated field at the objective with a bandwidth of 12–15 nm and a homogeneity of better than five per cent. It can jump to any wavelength between 260 and 680 nm in less than 3 ms and provides a 1.5 ms Fura jump between 340 and 380 nm. The modular design allows components to be easily added for complete imaging and photometric systems, as well as uncaging flashes, D/A converters and other accessories.

Reader Enquiry No. 102



FluoView for confocal fluorescence microscopes.

FluoView

From Olympus

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The system can collect and analyse 12-bit fluorescence and DIC images for all kinds of tissues, single cells and even subcellular structures. Configurations with the upright BX frame (including the fixed-stage, nose-piece focusing BX50WI) and the inverted optical-bench-designed IX frame incorporate the high-quality Olympus UIS optics and fluorescence performance. The microscope has been developed with completely integrated hardware and software. A modular scan unit comprises the point-scanning technique, based on two galvanometer mirrors. Two photomultiplier tubes are integrated for two-channel simultaneous image acquisition, and even three-channel, software-based sequential image detection. A single 488-nm line, argon laser and dual 488/569 nm line, krypton-argon lasers are standard.

Reader Enquiry No. 103

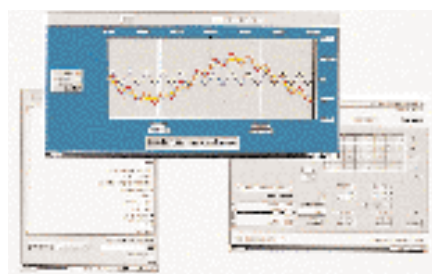
Data handling tools

NetAcquire Java Toolkit

From Real Time Integration

A toolkit that allows Java applets to acquire, process and update real-time analog and digital data over an Ethernet network

Java applets use the NetAcquire Java Toolkit and a TCP/IP network to communicate with NetAcquire server hardware containing analog-to-digital conversion hardware. NetAcquire is an 'intelligent' data acquisition



NetAcquire Java Toolkit applet for data handling.

system that makes analog and digital data available over an Ethernet network. NetAcquire extends the cross-platform architecture of Java 'write-once, run everywhere' systems by adding hardware input/output capabilities. Java is a hardware-independent environment, and the only way to interface to the 'real world' is using network-based peripherals, such as NetAcquire. The toolkit may prove to be a useful component for building enterprise-wide distributed test and measurement systems, as well as Internet-compatible systems that allow remote data acquisition and control from anywhere in the world. In conjunction with Netscape 3.0, the applets run on sixteen possible combinations of operating systems and computers. Both standalone Java applications and browser-enabled Java applets can connect to NetAcquire hardware to obtain a 'real-world' interface to analog and digital data.

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Lab-Lon

From Biodata

Instruments with RS232 ports can now be connected to a LonWorks network

Lab-Lon is designed for laboratories containing many instruments and enables a single personal computer to log data from all equipment. The small (120×60×40 mm) Lab-Lon adaptor plugs into the RS232 connector on the instrument. It uses a simple four-wire telephone cable to connect to the network. Another Lab-Lon links the network to the computer's serial port. Lab-Lon collects readings and checks whether they have changed since the last collection. If so, it passes the new data to the LonManager, with immediate notification of alarm conditions. High-level applica-



Biodata's Lab-Lon, a nexus between instruments and a LonWorks network through RS232 ports.

tions, such as LIMS packages, are no longer required to deal with the problem of asynchronous serial communications. Lab-Lon identifies the instrument in a consistent manner, so instruments can be moved around without having to worry about where they are on the network. Lab-Lon units can also be provided with a keyboard or bar-code entry facilities. According to the manufacturer, these units have been rigorously tested for six months at temperatures down to -60 °C, and have proved their reliability in harsh conditions.

Reader Enquiry No. 105

DasyLab

From Dasytec

Windows-based data acquisition software now available in 32-bit technology

The company now offers DasyLab versions 3.5 and 4.0, 16-bit and 32-bit Windows-based systems, respectively. Version 3.5 is a 16-bit software package that runs under all Windows systems; whereas, the 32-bit version 4.0 is designed for Windows 95 and NT, and is available with drivers for all major hardware manufacturers, as well as for RS232 and IEEE support. DasyLab turns a personal computer into a data logger, DSO, transient recorder or frequency analyser, with no programming required. The user simply selects a set of function icons for the desired task and connects them using the mouse. The system is stated to take minutes to set up and features analog and digital input and output, mathematical calculations, statistical functions, frequency analysis, programmable signal generators, and test and measurement control. Data display and analysis can be viewed in Windows as a chart recorder, a Y/t scope, an X/Y scope, a waterfall, a histogram, a bar graph, and digital and analog displays. DasyLab allows communication with other Windows applications using its DDE interface.

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Miscellaneous

Vecta series sample preparation

From Whatman

The series comprises centrifuge tube filters, a centrifuge tube and a centrifugal liquid extraction device

The VectaSpin centrifuge tube filters are designed to perform quick and easy filtration on a wide variety of samples. It is intended for use with the VectaSpin 3 centrifuge tube so that the filtrate can be stored after centrifugation using the same container. These devices should fit easily in common centrifuge rotors. The VectaSep CLE centrifugal liquid extraction device should enhance the preparation of aqueous samples in the analytical laboratory. Using this extraction

device, just two dispensing steps (solvent and sample) are required before extraction. The remaining steps are performed in minutes, according to the manufacturer, making the process simple, fast and trouble free. Sample extracts are typically obtained within 30–60 min of the dispensing steps. The VectaSep CLE has been designed for use with laboratory robots. The company's GD/X syringe filters make filtering viscous and hard-to-filter samples easier with a pre-filtration stack of media, which allows difficult samples to be filtered with less hand pressure than previously possible.

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